Mixed Matrix Membrane Chromatography for Bovine Whey Protein Fractionation

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Abstract

Whey protein fractionation is an important industrial process that requires effective large-scale processes. Although packed bed chromatography has been used extensively, it suffers from low processing rates due to high back-pressures generated at high flow rates. Batch chromatography has been applied but generally has a low efficiency. More recently, adsorptive membranes have shown great promise for large-scale protein purification, particularly from large-volume dilute feedstocks. A new method for producing versatile adsorptive membranes by combining membrane and chromatographic resin matrices has been developed but not previously applied to whey protein fractionation. In this work, a series of mixed matrix membranes (MMMs) were developed for membrane chromatography using ethylene vinyl alcohol (EVAL) based membranes and various types of adsorbent resin. The feasibility of MMM was tested in bovine whey protein fractionation processes.

Flat sheet anion exchange MMMs were cast using EVAL and crushed Lewatit® MP500 (Lanxess, Leverkusen, Germany) anion resin, expected to bind the acidic whey proteins β-lactoglobulin (β-Lac), α-lactalbumin (α-Lac) and bovine serum albumin (BSA). The MMM showed a static binding capacity of 120 mg β-Lac g^{-1} membrane (36 mg β-Lac mL^{-1} membrane) and 90 mg α-Lac g^{-1} membrane (27 mg α-Lac mL^{-1} membrane). It had a selective binding towards β-Lac in whey with a binding preference order of β-Lac > BSA > α-Lac. In batch whey fractionation, average binding capacities of 75.6 mg β-Lac g^{-1} membrane, 3.5 mg α-Lac g^{-1} membrane and 0.5 mg BSA g^{-1} membrane were achieved with a β-Lac elution recovery of around 80%.

Crushed SP Sepharose™ Fast Flow (GE Healthcare Technologies, Uppsala, Sweden) resin was used as an adsorbent particle in preparing cation exchange MMMs for lactoferrin (LF) recovery from whey. The static binding capacity of the cationic MMM was 384 mg LF g^{-1}membrane or 155 mg LF mL^{-1} membrane, exceeding the capacity of several commercial adsorptive membranes. Adsorption of lysozyme onto the embedded ion exchange resin was visualized by confocal laser scanning

microscopy. In LF isolation from whey, cross-flow operation was used to minimize membrane fouling and to enhance the protein binding capacity. LF recovery as high as of 91% with a high purity (as judged by the presence of a single band in gel electrophoresis) was achieved from 150 mL feed whey.

The MMM preparation concept was extended, for the first time, to produce a hydrophobic interaction membrane using crushed Phenyl Sepharose™ (GE Healthcare Technologies, Uppsala, Sweden) resin and tested for the feasibility in whey protein fractionation. Phenyl Sepharose MMM showed binding capacities of 20.54 mg mL^{-1} of β-Lac, 45.58 mg mL^{-1} of α-Lac, 38.65 mg mL^{-1} of BSA and 42.05 mg mL^{-1} of LF for a pure protein solution (binding capacity values given on a membrane volume basis). In flow through whey fractionation, the adsorption performance of the Phenyl Sepharose MMM was similar to the HiTrap™ Phenyl hydrophobic interaction chromatography column. However, in terms of processing speed and low pressure drop across the column, the benefits of using MMM over a packed bed column were clear.

A novel mixed mode interaction membrane was synthesized in a single membrane by incorporating a certain ratio of SP Sepharose cation resin and Lewatit MP500 anion resin into an EVAL base polymer solution. The mixed mode cation and anion membrane chromatography developed was able to bind basic and acidic proteins simultaneously from a solution. Furthermore, the ratio of the different types of adsorptive resin incorporated into the membrane matrix could be customised for protein recovery from a specific feedstream. The customized mixed mode MMM consisting of 42.5 wt% of MP500 anionic resin and 7.5 wt% SP Sepharose cationic resin showed a binding capacity of 7.16 mg α-Lac g^{-1} membrane, 11.40 mg LF g^{-1} membrane, 59.21 mg β-Lac g^{-1} membrane and 6.79 mg IgG g^{-1} membrane from batch fractionation of 1 mL LF-spiked whey. A tangential flow process using this membrane was predicted to be able to produce 125 g total whey protein per L membrane per h.

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Preface

The application of MMM chromatography for whey protein fractionation is the main subject of this study. Different types of MMM chromatography processes were developed for capturing various whey protein components. This study has produced several publications to date, as list below:

1. Syed M. Saufi and Conan J. Fee. (2009). Fractionation of β-lactoglobulin from whey by mixed matrix membrane ion exchange chromatography. *Biotechnology and Bioengineering*, **103**(1):138-147.

2. Syed M. Saufi and Conan J. Fee. (2009). Fractionation of whey proteins using anion exchange mixed matrix membrane (MMM) chromatography in a cross-flow system. The 7th International Conference on Membrane Science & Technology, 12-15 May. Kuala Lumpur, Malaysia.

3. Syed M. Saufi and Conan J. Fee. (2009). Recovery of lactoferrin from whey using cross-flow cation exchange mixed matrix membrane chromatography. Submitted to the *International Dairy Journal*.

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Symbols and Abbreviations

Symbols

c	equilibrium protein concentration, mg mL^{-1}
c_F	feed protein concentration, mg mL^{-1}
K, n	Freundlich constants
K_d	Langmuir dissociation constant, mg mL^{-1}
m	dry mass of the MMM
q	equilibrium protein binding capacities, mg protein bound g^{-1} adsorbent
q_{DBC}	dynamic binding capacity
q_m	maximum protein binding capacities, mg protein bound g^{-1} adsorbent
V_{BT}	permeate volume at 10% breakthrough
V_{dry}	volume of the dry membrane
V_{wet}	volume of the swollen membrane
ε	porosity

Abbreviations

AEX	anion exchanger membrane
ATPS	aqueous two phase systems
ATRP	atom transfer radical polymerization
BIB	2-bromoisobutyryl bromide
BOD	biological oxygen demand
BP	benzophenone
BSA	bovine serum albumin
CEW	chicken egg white
CEX	cation exchanger membrane
CLSM	confocal laser scanning microscopy
COD	chemical oxygen demand
CV	column volume
DEAE	diethylaminoethyl
DF	diafiltration
DI	deionized
DMSO	dimethysulfoxide

EDTA	ethylenediaminetetraacetic acid
EVAL	ethylene vinyl alcohol
FITC	fluorescein isothiocynate
GMP	glycomacropeptide
Hb	hemoglobulin
HCl	hydrochloric acid
HEMA	2-hydroxyethelmethacrylate
HIC	hydrophobic interaction chromatography
HIgG	human immunoglobulin
HPTFF	high performance tangential flow filtration
IgG	immunoglobulin G
Igs	immunoglobulins
LF	lactoferrin
LMH	L m^{-2} h^{-1}
LP	lactoperoxidase
LZY	lysozyme
MAET-MAC	2-(methacryloyloxy)ethyl-trimethylammonium chloride
MAPA	methacrylamidophenyalanine
MF	microfiltration
MMM	mixed matrix membrane
MPh	monophenyl trimethoxysilane
MW	molecular weight
NaCl	sodium chloride
NaOH	sodium hydoxide
Na_2SO_4	sodium sulphate
NF	nanofiltration
$(NH_4)_2SO_4$	ammonium sulphate
PES	polyethersulfone
poly(DMAEMA)	poly(2-dimethyl aminoethyl methacrylate)
PP	polypropylene
PSF	polysulfone
PVDF	polyvinylidene fluoride
Q	quaternary ammonium

RO	reverse osmosis
RPC	reverse phase chromatography
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	scanning electron microscope
SP	sulfopropyl
TFA	trifluoroacetic acid
TMP	transmembrane pressure
UF	ultrafiltration,
WPC	whey protein concentrate
WPI	whey protein isolate
α-Lac	α-lactalbumin
β-Lac	β-lactoglobulin

1 Introduction

1.1 Background

The interest of industry in producing new products or/and improving current products processing using biotechnology has raised new challenges for the separation engineer. Most products produced from a bioreactor contain many impurities that need to be separated. Sequential techniques and cost effective separations are required to reduce the overall cost because most of the bioproduction cost depends heavily on the purification process. The requirements for separation processes are more demanding if food or pharmaceutical products are involved, because they need much higher purity and have more stringent safety regulations than other industrial products.

Conventional techniques for bioproduct separation (bioseparation) involve several steps such as impurities removal, product isolation, product purification and polishing. Chromatography is a well-known unit operation that is involved in virtually all of the bioseparation steps mentioned above. In chromatography, the type of matrix and the principle of interaction between the matrix and the protein are important factors that must always be considered. This subject is important for chromatographic research internationally as efforts are made to produce efficient chromatographic systems for protein separation.

Currently, most chromatography systems use packed bed columns to contain the stationary phase or matrix. However several limitations have been identified within this system. Their weaknesses, such as high pressure drop, long processing times due to slow intraparticle diffusion and complicated scale up procedures (Ghosh 2002; Kawai et al. 2003; Klein 2000; Van Reis and Zydney 2001; Zou et al. 2001), have encouraged the search for new kinds of column material and configuration.

Membrane chromatography, which uses adsorptive membranes, is one of the alternatives to packed bed chromatography. Adsorptive membranes which have a specific functionality or ligand will bind to the target protein with different adsorption mechanisms depending on the type of ligand. Membrane chromatography

systems have shown distinct advantages such as (Ghosh 2002; Kawai et al. 2003; Wickramasinghe et al. 2006; Zou et al. 2001):

a) Low pressure drop across the column
b) Convective mass transfer properties of solutes to the binding sites
c) High flow rate operation with maintain performance
d) Easy column packing with low column clogging tendency
e) Uncomplicated scale up procedures
f) Low cost and the ability to be used as a single disposable device, especially for pharmaceutical or food applications which require very stringent cleaning protocols

Various types of membrane chromatography, which cover a wide range of applications have been reviewed by several authors (Charcosset 1998; Ghosh 2002; Kawai et al. 2003; Klein 2000; Saito et al. 1999). Both ion exchange and affinity interaction membrane chromatography processes have been widely reported and several ion exchange membranes are now commercially available from a variety of manufacturers, such as Sartorius, Vivascience, Millipore and Pall (Ghosh 2002). Most membranes chromatography are in flat sheet or radial flow configurations but lab-scale hollow fiber membranes have been developed by several authors for both cation (Camperi et al. 1999; Shinano et al. 1993; Tsuneda et al. 1994) and anion (Kubota et al. 1997) exchange.

Research efforts continue in the search for lower cost, easily prepared adsorptive membranes with high binding capacities. Three steps are usually involved in the preparation of adsorptive membranes: (1) preparation of the base membrane, (2) chemical activation of the base membrane and (3) coupling of ligands to the activated membrane (Zeng and Ruckenstein 1999). The latter two steps may require harsh chemical and physical conditions that can cause undesirable and irreversible changes in the membrane structure. The concept of mixed matrix membrane (MMM) chromatography, developed by Wessling's group (Avramescu et al. 2003), is an alternative that provides a simple method for the preparation of adsorptive membranes, yet, results in a high quality membrane chromatography performance.

MMMs are prepared by incorporating an ion exchange resin (or any adsorptive resin) into a membrane polymer solution prior to membrane casting. The chemical modifications necessary for incorporating adsorptive ligands are thus decoupled from the membrane preparation step. The availability of various low cost resins suitable for protein adsorption can make this technique cost effective.

Protein fractionation from milk or whey has become increasingly important during the past two decades as the dairy industry globally has moved from being solely based on commodity food production to earning a significant income from specialty proteins (Fee and Chand 2005; Fee and Chand 2006; Goodall et al. 2008). In view of the large volumes of whey that are produced every day in a milk processing plant, a high throughput chromatographic system such as membrane chromatography has significant potential for improving process efficiency (Splitt et al. 1996). Milk and whey protein separation using conventional packed bed chromatography is well known in the dairy industry; however membrane adsorber use began to expand in the industry only a few years ago. MMM chromatography has not previously been applied to whey protein fractionation. Most of the MMM prepared have been mainly applied to model protein mixtures and not many real feed streams have been tested. Therefore, the potential use of MMM for whey protein fractionation is the principal subject of this research, for which a range of MMM chromatography processes will be developed.

1.2 Objectives

The main objective of this research was to produce and characterise the performance of a range of membranes for MMM chromatography for potential use in whey protein fractionation. Different types of adsorptive MMM chromatography were developed with specific objectives as below:

1) Anion exchange MMM chromatography was developed by incorporating a Lewatit® MP500 (Lanxess, Leverkusen, Germany) anion exchanger resin into an ethylene vinyl alcohol (EVAL) base membrane. The anionic MMM was expected to bind acidic whey proteins such as β-lactoglobulin (β-Lac), α-lactalbumin (α-Lac) and bovine serum albumin (BSA).

2) A cation exchange MMM was developed using a SP Sepharose™ (GE Healthcare Technologies, Uppsala, Sweden) resin to recover lactoferrin (LF) from whey. The performance of the membrane was evaluated and the feasibility of using cross-flow filtration in the separation process was studied. In addition, the protein binding to the cation resin in the membrane matrix was visualized using a confocal laser scanning microscopy (CLSM) technique.

3) The concept of mixed matrix membrane chromatography was extended to the development of hydrophobic interaction MMM chromatography. Phenyl Sepharose™ (GE Healthcare Technologies) resin was selected as the adsorbent particle in the membrane matrix and was tested for whey protein fractionation. This is first time the concept of MMM chromatography was used to prepare a hydrophobic membrane chromatography material rather than ion exchange type membrane.

4) Mixed mode interaction membrane chromatography in a single membrane material was fabricated using the concept of MMM preparation. Before this, the mixed mode interaction in membrane was normally achieved by alternating different layers of membranes having different functionalities (anion and cation) in one module. The novel mixed mode interaction MMM chromatography was evaluated for separation of a model protein mixture as well as whey solution.

1.3 Thesis organization

Following the introduction in Chapter 1, relevant background for the current research is presented in Chapter 2. Thus, Chapter 2 reviews the properties and interest in whey protein fractionation. Several separation technologies conducted in past studies are summarized, emphasizing column based chromatography, conventional membrane filtration and membrane chromatography. In Chapter 3, the general experimental methodology is described. The results for each part of the major experimental work is then given in a specific chapter according to the type of MMM

developed. Chapter 4 describes the development of anion exchanger MMM from Lewatit MP500 for fractionation of acidic proteins from whey. The static and dynamic binding capacity of the membrane are measured and tested for whey fractionation in batch operation. Chapter 5 shows how a cation exchange MMM is fabricated using SP Sepharose resin. This cationic MMM was used for LF recovery in a cross-flow operation with different numbers of MMM layers inserted in a plate-and-frame membrane module to increase LF adsorption. Hydrophobic interaction MMM was developed using Phenyl Sepharose resin and this is described in Chapter 6. The possibility of using hydrophobic membranes in whey protein fractionation is also evaluated in Chapter 6. In Chapter 7, the development of mixed mode interaction MMM chromatography was studied. By combining anion and cation resins in a membrane matrix in different proportions, the mixed mode membrane was tested for different protein separations. Conclusions and recommendations for future work are presented in Chapter 8.

2 Literature review

2.1 Introduction

In this chapter two main topics will be reviewed. The first part relates to the background of membrane chromatography and the methods used for preparing adsorptive membranes. In the second part, the properties and applications of whey protein are described, followed by different processes for whey protein fractionation.

2.2 Membrane chromatography

Protein separation by packed bed column chromatography is very common in downstream processing. However, several limitations of packed bed column chromatography have been indentified such as a high pressure drop, relatively slow intra-bead mass transport, difficulty in column packing and complicated scale up procedure (Ghosh 2002; Kawai et al. 2003; Klein 2000; Van Reis and Zydney 2001; Zou et al. 2001). These limitations have led to the development of membrane chromatography technology.

Membrane chromatography, also known as membrane adsorption, uses an adsorptive membrane which carries specific functionality similar to a chromatography resin. This combines the principles of chromatography and membrane filtration in a single separation device. Normally, the pore size of the membrane chromatography material is in the range of microfiltration (MF) membranes. Besides having the advantages of membrane filtration in general, membrane chromatography may be preferred over column chromatography in terms of low pressure drop, high flow rate operation while maintaining adsorption capacity, convective mass transfer properties, low clogging tendency and easy column packing and scale up (Ghosh 2002; Kawai et al. 2003; Klein 2000; Van Reis and Zydney 2001; Zeng and Ruckenstein 1999; Zou et al. 2001).

However, the binding capacity of membrane chromatography for smaller proteins is sometimes lower than conventional gel-type chromatography media (Ghosh 2002; Wickramasinghe et al. 2006). One way to improve this capacity is by making a three-

dimensional hydrogel on the internal membrane surface, in the submicron range, where ligands are immobilized (Gebauer et al. 1997; Ventura et al. 2008). Large pore size distribution of membrane chromatography also influences the membrane capacity. The presence of pores that are too large could result in radial concentration gradients within the pores which in turn could lead to early breakthrough. This may result in the dynamic capacity being less than the static capacity (Wickramasinghe et al. 2006). Another potential limitation of membrane chromatography is non-uniform flow distribution across the membrane, due to the large diameter-to-length ratio of the modules (Charcosset 2006). However, with a proper design of flow distributors, this problem should be minimal (Ghosh and Wong 2006).

Similar to conventional chromatography media, different types of interaction are also possible for membrane chromatography, such as anion exchange, cation exchange, hydrophobic interaction and affinity interaction. In preparing MMM chromatography in the current study, ion exchange and hydrophobic interaction were exploited. Therefore, in the following section, various reaction schemes used to prepare different types of ion exchange and hydrophobic interaction membrane chromatography materials are reviewed. This is followed by a review of the literature related to the preparation and applications of MMM chromatography.

2.3 Preparation of ion exchange membranes

In order to produce high capacity ion exchange membranes, various preparation routes have been proposed. However, some of them are complicate and difficult to implement at large production scale. In the following paragraphs, a few recent examples for making anion and cation exchanger membranes will be described.

Surface-initiated atom transfer radical polymerization (ATRP) has been used to prepare anion exchange membranes from commercial MF base membrane. A two-step modification process was involved in preparing a weak anion exchange membrane from a regenerated cellulose membrane by Bhut et al. (2008). In the first step, initiator molecules, 2-bromoisobutyryl bromide (BIB) were anchored to the membrane pore surfaces. ATRP was used in the second step to grow poly(2-dimethyl aminoethyl methacrylate) (poly(DMAEMA)) chains on the initiator sites. The mass

of the poly(DMAEMA) grafted on the membrane surface was controlled by varying the polymerization time from 3 h to 12 h. For a membrane produced with a 12 h polymerization time, the maximum binding capacity for BSA was 66.3 mg mL^{-1}, which is about three times higher than commercial Sartobind® D membrane (Sartorius, Gottingen, Germany). In a later publication (Bhut and Husson 2009), the BIB initiator grafting density was increased by increasing the concentration of BIB in solution during the membrane initiator-functionalization reaction. The dynamic binding capacity of this membrane was measured between 80-90 mg BSA mL^{-1} for linear flow velocities in the range of 53-530 cm h^{-1} (Bhut and Husson 2009).

He and Ulbricht (2008) used a photo-grafting technique to prepare anion exchange membrane from a hydrophilized polypropylene (PP) MF membrane. The architecture of the grafted layer and grafting density could be controlled by using two different preparation routes, synergist immobilization or adsorption. In the adsorption method, hydrophilized PP membrane was soaked in benzophenone (BP) solution in the first step. Then, the pre-coated membrane was immersed in monomer solution of 2-(methacryloyloxy)ethyl-trimethylammonium chloride (MAET-MAC) saturated with BP, followed by15 min UV irradiation at a UV intensity around 6.5 mW cm^{-2}. For the synergist immobilization method, partial aminolysis of the thin hydrophilic polyacrylate layer was carried out as a pre-treatment in the initial step. The membrane was then immersed in a MAET-MAC monomer solution and subjected to UV irradiation treatment. To obtain a cross-linked grafted layer, a certain amount of cross-linker, N,N'-methylene-bis-acrylamide can be added in monomer solution in the synergist immobilization method. Based on the high grafting efficiency and better controllability, the synergist immobilization method was preferred for preparation of a well-defined membrane adsorber with a three-dimensional grafted layer (He and Ulbricht 2008).

Zhang et al. (2008) used electrospun cellulose acetate nanofibers that randomly overlaid into felts as a base membrane for making anion exchanger membrane. The diameters of the nanofibers were in the range from ten nanometers to microns and the pore sizes within the nanofiber felts were in the range from submicrons to microns. The cellulose acetate nanofiber felts were then converted into regenerated cellulose nanofiber felts by hydrolysis/deacetylation in a sodium hydroxide (NaOH)

aqueous solution. Subsequently, the regenerated cellulose nanofibers were treated with an aqueous solution containing 2-(diethylamino) ethyl chloride hydrochloride in an alkali condition at 90°C to introduce a diethylaminoethyl (DEAE) ligand onto the membrane. The adsorptive membrane felt showed a maximum binding capacity of 40 mg BSA g^{-1} membrane, which was higher than a commercial DEAE membrane adsorber which had a capacity of 33.5 mg BSA g^{-1} membrane.

Chiu et al. (2007) prepared a cation exchange membrane based on glass membranes for isolating lysozyme (LZY) from hen egg albumin. The glass membrane was first treated with piranha solution (70% H_2SO_4: 30% H_2O_2 (v/v)) at 95°C for 1 h to increase the density of silanol groups. The pre-treated membrane was then coated with a solution of monophenyl trimethoxysilane (MPh) in dimethylformamide and hydrochloric acid (HCl). Finally, the MPh-coated membrane was incubated in chlorosulfonic acid for 48 h to introduce sulfonic ligand on the membrane. For LZY isolation from egg albumin, the purification factor obtained by this cationic glass membrane was in the range 28.0-31.6 with the LZY recovery of 72.6-81.1% and purity of 78.9%.

Camperi et al. (1999) prepared a cation hollow fiber membrane by using an epoxy activated polysulfone based membrane. The hollow fiber membrane was incubated in a mixture of sodium sulfite/isopropyl alcohol/water (10 wt %/15 wt %/75 wt %) at 37 °C for 1-10 h. The remaining epoxy groups were then hydrolyzed into diol groups by treatment with 0.5 M sulfuric acid at 80 °C for 2 h. The cationic hollow fiber membrane showed a maximum binding capacity of 84 ± 9 mg LZY mL^{-1} and a dynamic binding capacity of 67% of the maximum capacity value.

A different approach was used by Fang et al. (2004) to prepare their cation exchange membrane. They modified polysulfone (PSF) powder to produce sulfonated PSF rather than modifying a prepared membrane. The sulfonation reaction was optimized in terms of reaction temperature and time. They also investigated the effects of membrane preparation conditions such as type of coagulation bath, relative humidity, exposure duration of gel-casted membrane and the ratio of additives to solvent in the polymer casting solution. The sulfonated cation exchange membrane showed a saturation capacity of 140 µg LZY cm^{-2} or 15.6 mg LZY mL^{-1} for experiments with

pure LZY at pH 7.4. For the isolation of LZY from hen egg white, a purification factor of 20.7 and a LZY recovery of 51.1% were obtained using one piece of the membrane under operation in dead-end filtration mode at 10 mL min^{-1} in a plate-and-frame module.

2.4 Preparation of hydrophobic interaction membranes

Based on the literature to date, the main separation chemistry utilized in membrane chromatography is ion exchange, followed by affinity interactions (Ghosh 2002). There are relatively few reports on hydrophobic interaction-based membrane chromatographic separations. Alkyl (e.g., butyl and octyl) and aryl (e.g., phenyl) groups are representative ligands for hydrophobic interaction chromatography (HIC). The number of hydrophobic amino acid side chains on the protein surface (such as valine, tryptophan, phenylalanine, leucine), and the ligand-polymer structure, the surface ligand density, the type and concentration of salt, pH of the medium and temperature influence the hydrophobic interaction separation of proteins. By manipulating separation process parameters, it is possible to enhance the hydrophobic interaction between the protein and hydrophobic ligand to affect the separation of protein molecules (Roper and Lightfoot 1995). In addition, HIC is an ideal "next step" after protein precipitation with ammonium sulphate ($(NH_4)_2SO_4$) or elution in high salt concentration during ion exchange chromatography.

Kubota's group, (Kubota et al. 1995; Kubota et al. 1996; Kubota et al. 1997a; Kubota et al. 1997b) is among the first groups involved in preparing hydrophobic interaction membrane chromatography. A series of polyethylene based hollow fiber HIC membrane was prepared by them. The polyethylene base membrane was modified with radiation-induced graft polymerization with glycidyl methacrylate monomer and attached with different types of hydrophobic ligand. HIC membranes were prepared at different ligand density and reaction conditions. Phenyl (Kubota et al. 1995; Kubota et al. 1997b) and butyl amine (Kubota et al. 1997a) membranes were prepared and tested with a single bovine serum albumin (BSA) protein. A binding capacity as high as 30 mg BSA g^{-1} membrane (Kubota et al. 1997a) was achieved, with repeated cycles of adsorption and elution being possible when 1 M NaOH regeneration was included between each cycle (Kubota et al. 1997b).

Arica et al. (2001) prepared HIC membranes with phenylalanine as a hydrophobic ligand. Two main reactions were involved. In the first reaction, phenylalanine was reacted with 2-methacrylochloride to produce a co-monomer of methacrylamidophenyalanine (MAPA). Then, a co-monomer of MAPA was reacted with 2-hydroxyethelmethacrylate (HEMA) monomer by UV-initiated photopolymerization at different HEMA/MAPA ratios to produce a flat sheet HIC membrane. The HIC membrane was tested for γ-globulins adsorption and showed a maximum adsorption capacity of 2.37 mg γ-globulins g^{-1} dry membrane.

Suen's group prepared an inorganic HIC membrane based on an alumina membrane (Chang and Suen 2006), modified with C8 and C18, and a glass fiber membrane (Chen et al. 2007), modified with a few types of short-chain organosilicon derivatives. Alumina HIC membrane however, had a low protein elution recovery using a salt-free elution buffer as normally applied to recover the bound protein from HIC. In order to recover the bound LZY and conalbumin from the alumina HIC membrane, 70% acetonitrile and 70% isoproponal were necessary, respectively, which are uncommon elution buffers for hydrophobic interaction chromatography because of their denaturing effects on the eluted proteins. Normally such buffers would only be used in reverse-phase chromatography, indicating that the membranes prepared were more hydrophobic than is normally consistent with HIC.

Commercial polyvinylidene fluoride (PVDF) MF membranes may also possibly perform in HIC in the presence of a high concentration $(NH_4)_2SO_4$ buffer, as demonstrated by Ghosh's group (Ghosh 2001; Ghosh 2005). A PDVF MF membrane was able to bind monoclonal antibody at 2 M $(NH_4)_2SO_4$ concentration (Ghosh 2001) and human immunoglobulin (HIgG) at 1.5 M $(NH_4)_2SO_4$ concentration (Ghosh 2005). More recently, it was found that this PVDF based HIC membrane could also be used to separate monoclonal antibody aggregates (i.e. monomer, dimmer, trimer etc.) very efficiently compared with size exclusion chromatography (Wang and Ghosh 2008; Wang et al. 2006). Based on the fact that the hydrophobicity of monoclonal antibodies increases with the degree of aggregation, a linear salt gradient elution in HIC membrane is able to discriminate different types of monoclonal antibody aggregates. Sartorius has expanded its membrane adsorber product range

recently by introducing a new Sartobind Phenyl membrane (Fraud et al. 2008) as a hydrophobic interaction membrane.

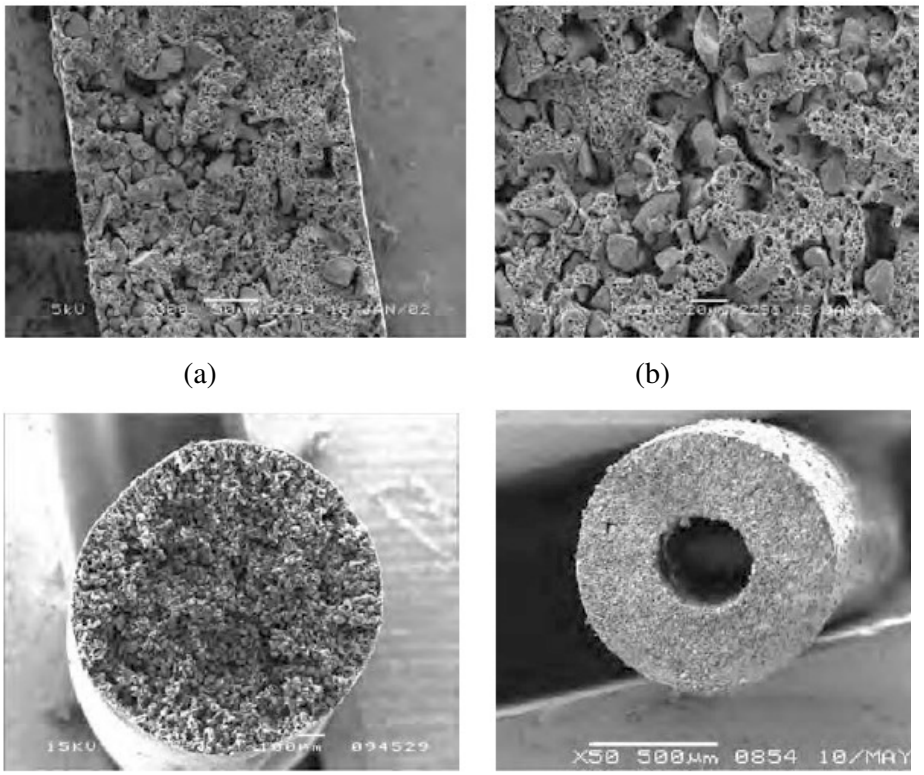
2.5 Mixed matrix membrane chromatography

It is believed that excessive or harsh chemical modification, as normally practiced in transforming a ready made membrane to membrane chromatography as described above, may unintentionally and irreversibly damage the membrane structure. The concept of a mixed matrix membrane preparation method was introduced by Wessling's group (Avramescu et al. 2003b; Avramescu et al. 2003c) in order to overcome the complicated routes for preparing membrane chromatography materials. MMMs are prepared by incorporating any adsorptive resin into a membrane polymer solution prior to membrane casting. The (chemical) incorporation of adsorptive ligands are thus decoupled from the membrane preparation step. This preparation concept provides a simple procedure and produces a competitive membrane chromatography performance. Different types of adsorptive resin may be used to produce various types of MMM to suit specific applications. Table 2-1 lists MMM prepared in the previous literature, with some selected images of MMMs shown in figure 2-1.

In the early development of MMM chromatography, Avramescu et al. (2003c) demonstrated that this preparation concept is flexible and offers the possibility to easily adjust the geometry, the adsorption capacity as well as the functionality of the membranes. They prepared various geometries of mixed matrix materials from an EVAL polymer and a Lewatit CNP80WS cation resin in the form of flat sheet, solid fiber and hollow fiber membranes. In static adsorption experiments, a protein capacity of 135 mg BSA g^{-1} membrane or 45 mg BSA mL^{-1} membrane was achieved. In a later publication, different types of resin were incorporated into the EVAL base membrane to prepare cation exchange MMMs by incorporating Lewatit SP112 resin and anion exchange MMMs by incorporating Lewatit MP500 resin (Avramescu et al. 2003b). They investigated the performance of these MMMs for separation of two similarly sized proteins, BSA and bovine hemoglobulin (Hb). By using a proper selection of the system pH, either anionic or cationic MMMs were able to separate the mixture of BSA and Hb effectively.

Table 2-1: Mixed matrix membrane chromatography prepared using different materials with various formats.

Authors	Membrane matrix	Adsorbent resin	Adsorbent loading, %	Configuration
Avramescu et al. 2008	Polyethersulfone	Lewatit CNP80WS - cation resin	50	Hollow fiber
Saiful et al. 2006	Ethylene vinyl alcohol	Lewatit CNP80WS - cation resin	65	Flat sheet
Zhang et al. 2006	Polyethersulfone	Lewatit CNP80WS - cation resin	10-85	Solid fiber
		Lewatit SP112WS - cation resin	50	
Kiyono et al. 2004	Polysulfone	Amberlite IR120 - cation resin	20-50	Hollow fiber
Avramescu et al. 2004	Ethylene vinyl alcohol	Lewatit SP112WS - cation resin	65	Flat sheet
Avramescu et al. 2003a	Ethylene vinyl alcohol	Lewatit SP112WS - cation resin	65	Flat sheet
		Lewatit K2629- cation resin		
Avramescu et al. 2003b	Ethylene vinyl alcohol	Lewatit SP112WS - cation resin	65	Flat sheet and solid fiber
		Lewatit MP500 - anion resin		
Avramescu et al. 2003c	Ethylene vinyl alcohol	Lewatit CNP80WS - cation resin	25-75	Flat sheet



(a) (b)

(c) (d)

Figure 2-1: Mixed matrix membrane chromatography in different geometries (a) flat membrane, (b) flat membrane, higher magnification, (c) full fiber, and (d) hollow fiber (adapted from Avramescu et al. 2009).

PSF and polyethersulfone (PES) have also been used as the base polymer in MMM preparation. Kiyono et al. (2004) prepared a hollow fiber MMM from a combination of a PSF base membrane and Amberlite® IR120 cation resin as the adsorbent particle in the matrix. This hollow fiber membrane was tested for diffusion dialysis using NaOH, NaCl and sodium sulphate (Na_2SO_4) solutions. More recently, Avramescu et al. (2008) incorporated Lewatit CNP80WS into a PES based membrane matrix to prepare a cationic hollow fiber MMM. This hollow fiber MMM showed a static binding capacity of 210 mg LZY g^{-1} membrane or 60 mg LZY mL^{-1} membrane from a pure protein solution and was used to recover LZY from chicken egg white (CEW). The LZY concentration increased from about 3% in the feed solution to around 83% in the desorption buffer after a single adsorption-desorption cycle for LZY isolation from CEW. This gives a purification factor of about 27.

Most of the MMM prepared have been mainly applied to a model protein mixture. There are still lack of applications on MMM's for real feed streams and in particular no examples of whey protein fractionation has been conducted in the past. In whey protein fractionation, low-cost and effective membranes are required for isolation of food-grade proteins (which have a much lower value than many of the pharmaceutical-grade) which normally purified by expensive column chromatography. Also, hydrophobic interaction and mixed mode adsorption (particularly customized for a specific feed composition, such as whey) have not been demonstrated with MMM's. Therefore there are lot of opportunities exist in whey protein fractionation using MMM.

2.6 Whey protein

Whey is the liquid byproduct of casein precipitation of milk in the cheese or casein manufacturing industries and of milk concentration prior to milk powder production. Depending on the quality of the milk, 10 kg of milk will produce only 1-2 kg of cheese, while the rest of it will emerge as liquid whey (Bhattacharjee et al. 2006). Enormous quantities of whey are produced annually worldwide. The world production of cheese whey per year is estimated at 130 million tons, accounting for around 780,000 tons of protein (Monteiro et al. 2008). Figure 2-2 shows the main components of bovine milk, where whey protein only represents about 20% of the total protein in milk. The detailed composition and properties of individual whey proteins are given in table 2-2.

The disposal of whey, having a biological oxygen demand (BOD) value of about 35-60 g L^{-1} and a chemical oxygen demand (COD) value of 80-100 g L^{-1} as sewage, causes severe environmental pollution problems (Bhattacharjee et al. 2006). Government and other regulatory authorities in most countries have restricted or banned the disposal of untreated whey (Smithers 2008). This legislative restriction on whey disposal encouraged a deeper exploration of whey protein-based ingredients.

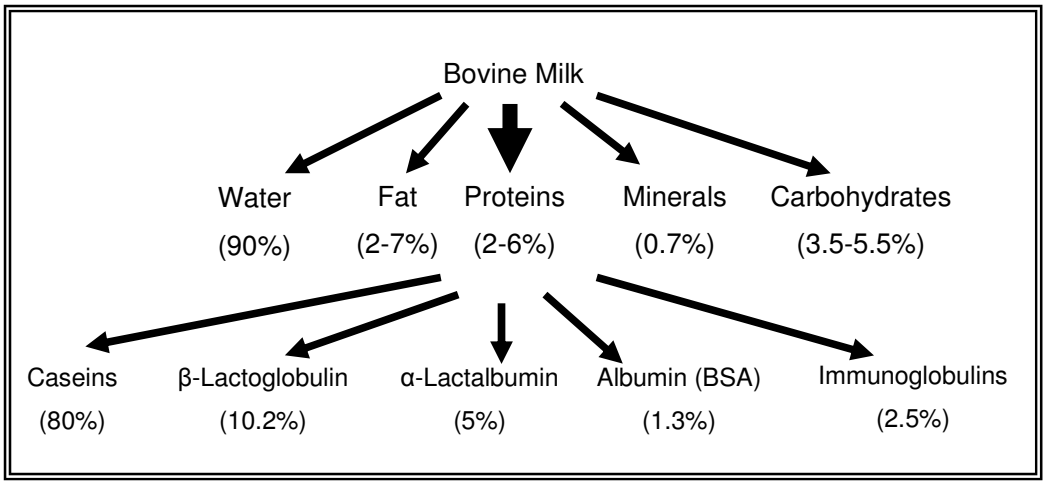


Figure 2-2: Main components of bovine milk and composition of bovine protein (Splitt et al. 1996).

Table 2-2: Whey protein composition (Andersson and Mattiasson 2006).

Protein	Concentration (mg mL^{-1})	Molecular weight (kDa)	Isoelectric point
β-Lactoglobulin	2-4	18	5.2
α-Lactalbumin	1.2-1.5	14	4.5-4.8
Immunoglobulins	0.6-0.9	150-1000	5.5-8.3
Bovine serum albumin	0.3-0.6	69	4.7-4.9
Lactoferrin	0.02-0.2	78-92	8-9.5
Lactoperoxidase	0.02-0.05	78-89	9.5

For bulk use, whey protein can be converted into whey protein concentrate (WPC) (35-80% protein) or whey protein isolate (WPI) (80-95% protein) (Brans et al. 2004). WPC is a mixture of all whey proteins obtained by membrane concentration of whey, retaining significant amounts of the minerals, lipids, and lactose from whey. WPI is a higher quality and higher value mixture of the whey proteins, manufactured using either membrane filtration or ion-exchange adsorption (Turhan and Etzel 2004). WPI is normally included in sports formulas, infant formulas or medical formulas, mainly

due to its high protein content. It also can used as a source of pure proteins or can be hydrolysed to obtain valuable peptides (Lucena et al. 2007).

In the past two decades, the dairy industry globally has moved from being based solely on commodity food production to earning a significant income from individual proteins from whey (Fee and Chand 2006; Horton 1995; Huffman and Harper 1999). With advances in science and technology, the added value of whey has increased dramatically over the past 50 years, as depicted in figure 2-3 (adapted from Smithers 2008). Whey components, particularly proteins and peptides, will increasingly be preferred as ingredients for functional foods and nutraceuticals and as active medicinal agents (Smithers 2008).

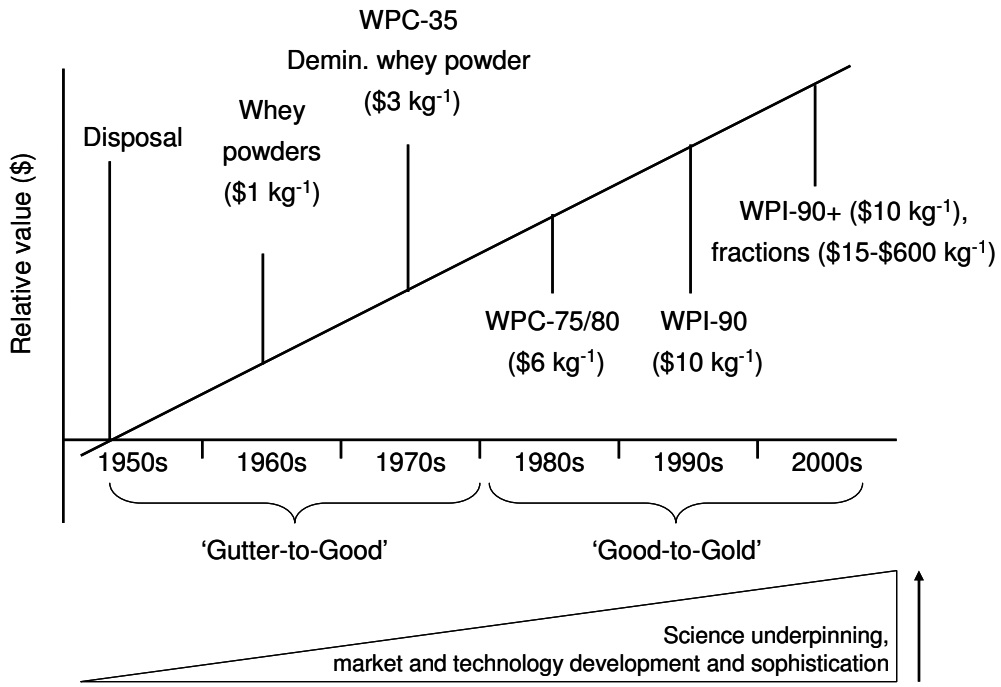


Figure 2-3: Schematic representation of the relative increase in value of whey protein/peptide products with increasing underpinning scientific knowledge of whey solids and advances in technology and marketplace sophistication over the past approximately 50 years (adapted from Smithers 2008).

However, the unique nutritional, therapeutic and functional characteristics of the individual whey proteins are largely unrealized due to interactions between components and degradation during processing. Thus, there is a challenge in whey protein fractionation to produce individual whey proteins with well characterized functional and biological properties by a process which will not denature but retain its nutritional and other properties (Bhattacharjee et al. 2006). In the following section, the properties and applications of the main whey protein components are described. (Note: unless otherwise stated, the information below relates to bovine milk and whey – the protein content in the milk and whey of other species, notably humans, differs significantly).

2.7 Whey protein components

2.7.1 β-Lactoglobulin – properties and applications

β-Lac is the major protein in bovine whey, although, importantly, it is virtually absent from human milk and whey. It represents approximately 58% of whey protein or 10% of total milk protein (Lozano et al. 2008). The concentration of β-Lac in whey is in the range of 2-4 mg mL^{-1} (Andersson and Mattiasson 2006). The primary structure of β-Lac consists of 162 amino acids with a molecular weight (MW) of approximately 18.4 kDa. Six genetic variants of bovine β-Lac are known so far, with the most common being the A and B variants. β-Lac A and B variants differ only at two positions of amino acid residue, 64 and 118, which are Asp and Val for β-Lac A and Gly and Ala for β-Lac B (Elofsson et al. 1997). Because β-Lac A has one more negative charge in terms of the amino acid compositions, it has slightly lower pI value (pI 5.1) than β-Lac B (pI 5.2) although the MW are essentially the same (Yamamoto and Ishihara 1999).

The secondary structure of β-Lac comprises nine strands of β structure, a short α helix segment and three helicoidal turns. Its quaternary structure depends on the medium pH: it occurs mainly as a stable dimer, with a MW of 36.7 kDa, at pH values between 5.2 and 7; as an octamer, with a MW of 140 kDa, at pH values between 3.5 and 5.2; and as a monomer, with two-cysteine residues per monomer, at pH 3.0 and above 8.0 (de Wit 1989).

β-Lac is a good source of essential amino acids and has a potential use in power drinks due to its good solubility (Horton 1995). Good gelling formation and better foam stabilizer compared to other whey proteins would make β-Lac suitable for confection production (Cowan and Ritchie 2007; Zydney 1998). Some of the biological functions of β-Lac that were identified by previous publications was summarized by Madureira et al. (2007). β-Lac plays a role in regulation of mammary gland phosphorus metabolism, transporter for vitamin D, cholesterol and retinol, transfer of passive immunity to the newborn and enhancement of pregrastic esterase activity (Madureira et al. 2007).

The β-Lac content of bovine milk is much higher than in human milk (El-Agamy 2007; Fox and McSweeney 1998) and this has been identified as a potential source of allergic reactions to infant formulae seen in some children (El-Agamy 2007; Monaci et al. 2006; Suutari et al. 2006). Therefore, the removal of β-Lac from whey would broaden the applications of whey products derivatives in the food industry (Casal et al. 2006). β-Lac free whey would also serve as the primary protein constituent of hypoallergenic infant formulas with protein compositions that are more similar to that of human milk (Casal et al. 2006).

2.7.2 α-Lactalbumin – properties and applications

α-Lac is the second largest protein component in bovine milk, comprising approximately 3.4% of total milk protein or 20% of the whey protein. The concentration of α-Lac in whey protein is in the range of 1.2-1.5 mg mL^{-1} (Andersson and Mattiasson 2006). On the other hand, α-Lac is the predominant whey protein in human milk with a concentration of 2.44 ± 0.64 mg mL^{-1} (after 30 days of lactation), as determined by Jackson et al. (2004).

α-Lac is a strong Ca^{2+} binding protein with four disulphide bonds consisting of 123 amino acids in a single peptide chain. Its MW is about 14.2 kDa with a pI of 4.2. At the amino acid level, the homology between human and bovine α-Lac can be described as having 76% fully conserved residues (93 out of 123 amino acids) (Chatterton et al. 2006).

α-Lac is the preferred protein source for infant formulas due to its high tryptophan content, high digestibility, and lower potential for causing allergies, when compared to β-Lac (Gurgel et al. 2000; Zydney 1998). Besides that, owing to its high content in tryptophan it is applicable as a nutraceutical and due to its high cytotoxicity it possesses therapeutic uses (Konrad and Kleinschmidt 2008). α-Lac also has strong affinity for glycosylated receptors on the surface of oocytes and spermatozoids and may thus have potential as a contraceptive agent (Zydney 1998). Its biological functions reported were included as a cancer preventer, lactose synthesis and treatment of chronic stress-induced disease (Madureira et al. 2007).

2.7.3 Bovine serum albumin – properties and applications

BSA exists in whey at concentration between 0.3-0.6 mg mL^{-1} (Andersson and Mattiasson 2006). The MW of BSA is about 69 kDa and it has a pI of around 4.7-4.9 (Andersson and Mattiasson 2006). It consists of 582 amino acid residues with 17 intramolecular disulfide bonds and a single free thiol (Burr 2001).

Some biological functions of BSA are fatty acid binding, anti mutagenic function and cancer prevention (Madureira et al. 2007). BSA also has a good gelling properties (Matsudomi et al. 1991) and it is also widely used in food and therapeutic applications (Zydney 1998).

2.7.4 Lactoferrin – properties and applications

LF is glycoprotein and a member of a transferrin family, which is capable in binding and transferring iron (Adlerova et al. 2008; Levay and Viljoen 1995). The concentration of LF in bovine milk and whey is relatively low and varies throughout the milking season, generally falling within the range 20-400 mg L^{-1} (Palmano and Elgar 2002). On the other hand, the concentration of LF in human milk is around 1000-3000 mg L^{-1} (Wakabayashi et al. 2006).

LF comprised of a single polypeptide chain containing 703 amino acids folded into two globular lobes, each with one iron binding sites. There are three forms of LF

according to its iron saturation: apolactoferrin (iron free), monoferric form (one ferric ion), and hololactoferrin (binds two Fe^{3+} ions) (Adlerova et al. 2008; Levay and Viljoen 1995). Besides iron, LF is capable of binding a large amount of other compounds and substances such as lipopolysacharides, heparin, glycosaminoglycans, DNA, or other metal ions like Al^{3+}, Ga^{3+}, Mn^{3+}, Co^{3+}, Cu^{2+}, Zn^{2+} etc. However, its affinity for these other ions is much lower (Adlerova et al. 2008).

LF has diverse bioactivity, including broad spectrum antimicrobial activity, promotion of iron transfer and absorption, cancer prevention, cell proliferation and differentiation, antiviral, antibacterial and antiparasitic activity (Adlerova et al. 2008; Horton 1995; Lu et al. 2007; Tomita et al. 2009; Wakabayashi et al. 2006). It was proved by many scientific findings that oral administration of LF exerts beneficial effects on the health of humans and animal, including anti-infective, anticancer and anti-inflammatory effects (Wakabayashi et al. 2006; Yamauchi et al. 2006). LF also has been commercially used as a natural bioactive ingredient in supplement foods (e.g. infant formulae and dietary supplement tablets), skin care and oral health care products (Wakabayashi et al. 2006). More recently, the discovery of LF, an antimicrobial peptide produced from pepsin digestion of lactoferrin, which showed a similar function as LF, attracted more interest in the exploration of LF application (Tomita et al. 2009).

2.7.5 Immunoglobulins – properties and applications

Immunoglobulins (Igs) are very complex proteins which consist of two long (heavy) and two shorter (light) polypeptide chains linked by disulphides bond. There are five classes of Ig: IgA, IgG, IgD, IgE and IgM. Only IgG, IgA and IgM present in milk. IgG can be further divided into a subclasses of IgG1 and IgG2. The principal Ig in bovine milk is IgG1, while in human milk it is IgA (Fox and McSweeney 1998). Table 2-3 shows the concentration of Ig found in bovine and human serum and mammary secretions. Detailed structures of different Ig classes can be found in the literature (El-Loly 2007; Fox and McSweeney 1998).

Table 2-3: Immunoglobulin concentrations in bovine and human serum and mammary secretions (Hurley 2003).

Species	Immunoglobulin	Concentration, mg mL^{-1}		
		Blood serum	Colostrum	Milk
Bovine	Total IgG	25.0	32-212	0.72
	IgG1	14.0	20-200	0.60
	IgG2	11.0	12.0	0.12
	IgA	0.4	3.5	0.13
	IgM	3.1	8.7	0.04
Human	Total IgG	12.1	0.4	0.04
	IgA	2.5	17.4	1.00
	IgM	0.9	1.6	0.10

In addition to antigen binding, all Igs exhibit one or more effectors functions, which are capable of preventing the adhesion of microbes to surfaces, inhibiting bacterial metabolism by blocking enzymes, agglutinating bacteria and neutralizing toxins and viruses (El-Loly 2007). Addition of bovine Igs in infant formulae and other foods may help to reduce viral and microbial infections and may provide consumers with improved immune activity (Gapper et al. 2007). In fact, the specific concentration of Ig can be raised in colostrum or milk by immunizing cows with its pathogen or antigen. This technique was used to produce hyperimmune colostral or milk products. A few commercial hyperimmune milk products are already on market and more applications can be expected in the coming years (Mehra et al. 2006).

2.7.6 Lactoperoxidase – properties and applications

Lactoperoxidase (LP) is among the most abundant enzymes found in milk protein. The concentration of LP in bovine milk is about 30 mg L^{-1} constituting about 1% of the whey proteins, while in human milk it is only 5% of that in bovine milk (Shakeel ur et al. 2002). LP has a MW of 78-89 kDa (Andersson and Mattiasson 2006) with 612 amino acid residues (Seifu et al. 2005) and pI value around 9.6 (Andersson and Mattiasson 2006). The levels of iron and carbohydrate in LP are 0.068-0.079% and

9.9-10.2%, respectively (Shakeel ur et al. 2002). The secondary structure of LP consists of 65% β-structure, 23% α-helix and 12% uncoordinated structures (Shakeel ur et al. 2002).

LP catalyzes the oxidation of thiocynate by hydrogen peroxide and generates intermediate products with antibacterial properties which kill or inhibit the growth of wide range of bacteria, viruses, fungi, molds and protozoa (Korhonen 2009; Seifu et al. 2005; Shakeel ur et al. 2002). LP has also been used to preserve raw milk quality during transportation from the farmer to the dairy plant in the area where is not possible to used mechanical refrigeration (Seifu et al. 2005). In the following section, several major techniques for whey protein fractionation will summarized and highlighted.

2.8 Column based chromatography for whey protein fractionation

Chromatography is a very well known unit operation in downstream processing of protein mixture. In chromatographic techniques, the principle separation occurs due to the different migration of the component of interest between the stationary phase (i.e. matrix phase) and continuous phase (i.e. solvent) in the system. Chromatography media (i.e. stationary phase) is normally packed into a column from several centimeters to several meters diameter, depending on the process scale. Various types of chromatography mode or interaction are available, such as size exclusion, ion exchange, hydrophobic interaction and reverse phase chromatography. They differ in terms of the separation mechanism and selection of stationary and continuous phase. Table 2-4 gives a brief performance comparison between several types of chromatographic interaction that are normally used in column chromatography (Chaga 2001; Suen et al. 2003). The advantages and disadvantages between different formats of stationary phase in chromatographic system are given in table 2-5 (Ghosh 2003).

Previous studies on whey fractionation using column chromatography are summarized in table 2-6. Most of them appeared in the literature for the past 10

Table 2-4: Comparison between different types of column chromatographic mode (Chaga 2001; Suen et al. 2003).

Property	Affinity		Ion exchange	HIC/RPC[1]
	Group-specific	Bio-specific		
Adsorption capacity	Medium-high	Low	High	Medium-high
Selectivity	Medium-high	High	Low-medium	Low-medium
Recovery	High	Medium	High	Medium
Loading condition	Mild	Mild	Mild	Sometimes harsh
Elution condition	Mild	Harsh	Mild	Mild
Regeneration	Complete	Sometimes incomplete	Complete	Incomplete
Cost	Low	High	Low	Low

[1] HIC- hydrophobic interaction chromatography, RPC- reversed-phase chromatography

Table 2-5: Advantages and disadvantages of several types of stationary matrix in chromatography process (Ghosh 2003).

Processes	Advantages	Disadvantages
Packed bed	Establish process High resolution High binding capacity Suitable for gradient chromatography	Slow process Low reproducibility (with soft gels) Susceptible to column blinding Variability of column packing
Monolith	Fast High reproducibility Suitable for gradient chromatography Predominance of convective transport Low pressure drop	Expensive
Membrane	Fast High reproducibility Predominance of convective transport Low pressure drop Inexpensive Disposable device	Unsuitable for gradient chromatography Low binding capacity
Fluidized or expanded bed	Fast Suitable for feed containing particles	Poor resolution Poor reproducibility High energy consumption Breakage of chromatographic media

Table 2-6: Selected chromatography technique for whey protein fractionation in the past 10 years.

Author	Protein of interest	Protein source	Materials/configuration	Mode of interaction/ Ligand
Brochier et al. 2008	β-Lac	microfiltered whey	HyperCel™ column (Pall BioSepra), column volume - 2.5 mL, 5 mL, 10 mL	mixed mode - hexyl amine
Etzel et al. 2008	WPI	whey	Mono™ S column (GE Healthcare Technologies), column volume - 2.38 L, 10 cm diameter	cation exchange – methyl sulphonate
Etzel et al. 2008	WPI	whey	SP Sepharose Big Beads™ (GE Healthcare Technologies), column volume - 5.34 L, 20 cm diameter , 17 cm height	cation exchange - SP
Liang et al. 2006	β-Lac, α-Lac, BSA, IgG	whey	Sephadex™ G-200 (GE Healthcare Technologies); 2.6 cm × 70 cm	gel filtration
Fee and Chand 2006	LF, LP	milk	SP Sepharose Big Beads, column volume - 5 mL	cation exchange - SP
Schlatterer et al. 2004	β-Lac	whey	Macro-Prep ceramic hydroxyapatite (BioRad), column dimension 12 mm × 88 mm	-
Turhan and Etzel 2004	α-Lac, WPI	lactic acid whey	SP Sepharose Big Beads, column volume - 80 mL	cation exchange - SP
Rojas et al. 2004	α-Lac, β-Lac	protein fraction from ATPS	Sephadex G-25 HR-10/10 (GE Healthcare Technologies)	gel filtration

Table 2-6 continued

Doultani et al. 2004	α-Lac, WPI, LP, LF	whey	SP Sepharose Big Beads, column volume - 80 mL	cation exchange - SP
Neyestani et al. 2003	β-Lac, α-Lac, BSA	whey	1) Sephadex G-50 (GE Healthcare Technologies), column volume - 131 mL, dimension 1.6 cm × 65 cm 2) DEAE column (GE Healthcare Technologies), column volume - 5 mL	size exclusion and anion exchange - DEAE
Vyas et al. 2002	β-Lac	whey	Calcium bio-silicate particles	affinity - all-trans-retinal ligand
Gurgel et al. 2001	α-Lac	WPI	Polyhydroxylated methacrylate - TosoHaas AF Chelate 650	affinity - peptide ligand
Tellez and Cole 2000	β-Lac, α-Lac, BSA, IgG	whey	Biogel™ A 0.5 m and 5 m (Bio-Rad); column 1- dimension 1.5 cm × 30 cm, column 2- dimension 2.5 cm × 60 cm	electrochromatography
Ye et al. 2000	α-Lac, β-Lac A, β-Lac B, LP, LF	whey	1) SP-Toyopearl™ (Toyosoda), dimension 1.5 cm × 18 cm 2) Quaternary aminoethyl-Toyopearl (Toyosoda), dimension 1.5 cm × 18 cm	anion and cation exchange

Abbreviation: WPI - whey protein isolates, α-Lac - α-lactalbumin, β-Lac - β-lactoglobulin, BSA - bovine serum albumin, LF - lactoferrin, LP - lactoperoxidase, IgG - immunoglobulin G, SP - sulfopropyl, DEAE - diethylaminoethyl, ATPS - aqueous two phase systems.

years. Detailed descriptions of selected processes are elaborated in the following paragraph.

Column chromatography used for whey protein fractionation is dominated by ion exchange chromatography. In whey protein fractionation, either selective adsorption or selective elution can be performed. In selective adsorption, whey protein of interest is adsorbed onto the column while leaving a whey solution depleted in that protein. In selective elution, all the whey proteins are trapped simultaneously onto an adsorbent, rinsed free of contaminants and then eluted one-by-one (Almecija et al. 2007).

Doultani et al. (2004) used selective elution method to recover different fractions of whey protein that bound onto an SP Sepharose Big Beads (GE Healthcare Technologies), cation exchanger column at pH 4. Different elution sequences can be used to recover the bound whey protein to produce the following fractions:

(1) Single WPI by using 10 mM NaOH
(2) α-Lac and WPI depleted in α-Lac fraction by using 100 sodium acetate pH 4.9 and 10 mM NaOH elution buffer respectively
(3) α-Lac, WPI depleted in α-Lac, LP and LF by using this elution sequence: 100 sodium acetate pH 4.9, 50 mM sodium phosphate pH 6.5, 0.35 M NaCl in 50 mM sodium phosphate pH 6.5 and 1.20 M NaCl in 50 mM sodium phosphate pH 6.5

According to them, this method offers a flexibility to switch between different protein fractions day-to-day depending on the market and customer demands.

This selective elution was also successfully applied for producing α-Lac and WPI depleted in α-Lac fraction by Turhan and Etzel (2004) using the same chromatographic column. The purity of α-Lac achieved in this study was 93% while the WPI depleted in α-Lac contains less than 2% α-Lac. Ye et al. (2000) used a salt gradient for selective elution of whey protein by using two different types of ion exchanger columns. Whey at pH 6.5 was passed through into first cation exchanger column (SP-Toyopearl) to bind LP and LF. These proteins were eluted from a cation column using salt gradient of 0-0.55 M NaCl in 50 mM Tris-HCl buffer at pH 6.5.

The unbound whey solution that previously passed through a cation column was adjusted to pH 8.5 to feed into a second anion exchanger column (quaternary aminoethyl-Toyopearl) to bind α-Lac and β-Lac. Bound α-Lac was eluted from the anion column using 0-0.15 M NaCl in 50 mM Tris–HCl, pH 8.5. Next, the column was adjusted to pH 6.8, and β-Lac was eluted using salt gradient of 0-0.20 M NaCl in 50 mM Tris–HCl, pH 6.8. However, this method did not produce IgG and BSA fractions.

Neyestani et al. (2003) used a series of chromatography steps to obtain pure β-Lac from whey after precipitation with 50% $(NH_4)_2SO_4$. Both precipitate and supernatant obtained were dialyzed and lyophilized for further separation by chromatographic method. Lyophilized precipitate fraction was reconstituted in distilled water and run onto gel filtration column (Sephadex G-50, 131 mL column volume, 65 cm length) to obtain a first peak of the mixture of BSA and casein, and second peak of pure β-Lac. The yield of β-Lac was 166 mg based on 50 mL of starting milk. Meanwhile, lyophilized supernatant was dissolved in water and injected to DEAE column. Stepwise elution was applied onto the column resulted in two separate peak; a mixture of BSA and α-Lac in the first peak and single β-Lac in the latter peak. The yield of β-Lac was estimated about 178 mg β-Lac per 50 mL of milk for the second peak. The mixture of BSA and α-Lac was further applied to gel filtration column to separate a single protein fraction. The yield of BSA and α-Lac based on 50 mL milk was calculated about 11.5 mg and 54.5 mg respectively from the resolved peak.

Besides packed bed columns, whey protein fractionation can also be operated in batch adsorption or fluidized (expanded) bed chromatography. de Jongh et al. (2001) used a batch process to isolate β-Lac using DEAE sepharose resin. Whey was diluted with water to a low conductivity value of 6.7 mS at pH 7.2. At this conductivity value, almost 80-90% β-Lac could be bound from 1 L whey by 77 g of resin used. The bound β-Lac was eluted by 0.25 M NaCl and this fraction was desalted by 10 kDa UF membrane. Final purification was achieved using Superdex 75 gel filtration column, followed by UF concentration to achieve a final β-Lac with purity > 98%. The yield of β-Lac production from 1 L was calculated higher than 80%. When 25 L whey was used, 50 g of β-Lac could be produced and the yield of the process dropped to a value between 65-70%.

Vyas et al. (2002) compared the performance of three different operating systems (packed column, stirred tank and fluidized bed column) for isolating β-Lac using affinity chromatography. The ligand used was all-trans-retinal immobilized on calcium bio-silicate resin. The packed column, stirred tank and fluidized bed produced β-Lac with purity of 80%, >95% and >95% and recovery of 0.65, 2.88, and 2.88 g β-Lac kg^{-1} of resin respectively. The fluidized bed column was considered best suited for industrial point of view and scale up. Noppe et al. (1999) directly isolated α-Lac from defatted bovine and goat milk using hydrophobic expanded bed chromatography. Upon Ca^{2+} removal by ethylenediaminetetraacetic acid (EDTA) presented in the buffer, α-Lac underwent a significant conformation change rendering it more hydrophobic. At this conformation, it can selectively bind to Streamline® Phenyl (GE Healthcare Technologies) hydrophobic resin. Pure α-Lac was recovered from defatted goat milk, while α-Lac from bovine milk was contaminated by small amount of γ-casein. This casein can easily be removed by further size exclusion chromatography if necessary to achieve a pure α-Lac.

Some interesting techniques in whey protein fractionation using chromatography methods had also been made in recent years. Fee and Chand (2006) successfully demonstrated that it is possible to extract LF and LP directly from the raw milk without affecting the gross properties of milk (fat content, protein content). They passed raw milk through a SP Sepharose Big Beads column by controlling the temperature at around the milking temperature 35-37°C. At this temperature, fat was believed to soften and did not block the column, so over 100 column volumes of milk were able to flow through. The dynamic binding capacity of the column was 48 mg mL^{-1} for LF and 0.55 mg mL^{-1} for LP. Brochier et al. (2008) demonstrated the feasibility of using a mixed-mode chromatography column for isolation of β-Lac from whey without the need for pH or conductivity adjustment using a hexyl amine mixed mode column (HyperCel™, Pall BioSepra, Cergy, France). A smooth scalability from 2.5 mL to a 10 mL column volume (CV) was achieved to extract all β-Lac content from 5CV of whey loaded into the column. Bound β-Lac was eluted at pH 4 with the purity was estimated to be around 75%. Meanwhile, Schalatterer et al. (2004) used ceramic hydroxyapatite column (Macro-Prep, BioRad, Munich, Germany) to isolate β-Lac from acid whey originating from milk of healthy and

mastitic cows. A single peak of β-Lac could be eluted at a sodium fluoride concentration of 0.6 M. Using whey from a healthy cow, the yield of β-Lac around 50-55% with purity more than 96% was achieved. For mastitic whey, the yield of β-Lac was between 18-20% with low purity, contaminated by IgG, BSA and LF.

Most of the latest studies related to the whey protein fractionation by chromatographic techniques described above are still based on a lab scale production. Further challenge in scale up procedures will be involved for pilot plant development. Compared with packed bed columns, expanded bed chromatography shows a benefit for direct capture of whey protein without requiring whey pre-filtration. However, a lack of studies has been done so far for this kind of chromatography for whey protein fractionation.

2.9 Conventional membrane filtration for whey protein fractionation

Membrane processes are well known in dairy processing plants. They are extensively used in the separation of fat globules in cream manufacturing, reduction of bacteria and spores in skim milk and concentration of casein micelles as a pre-treatment in cheese manufacturing (Brans et al. 2004; Pouliot 2008). Ultrafiltration (UF) is normally used for preparing WPC or is combined with dialfiltration (DF) in producing WPI (Zydney 1998). Figure 2-4 matches common membrane process with the components typically found in milk which are based on size ranges (Brans et al. 2004). However, full milk fractionation is very challenging due to the broad particle size distribution of milk component and various concentration levels for each component in milk.

Generally, in size-based separation process, the size difference between the components to be separated should be at least a factor of ten. Single whey protein fractionation by conventional membrane filtration (UF particularly) is impossible due to the similar size of whey protein components. Using a few stages of membrane module or combination with other separation processes is possible, however high costs and complicated operational procedures will be involved.

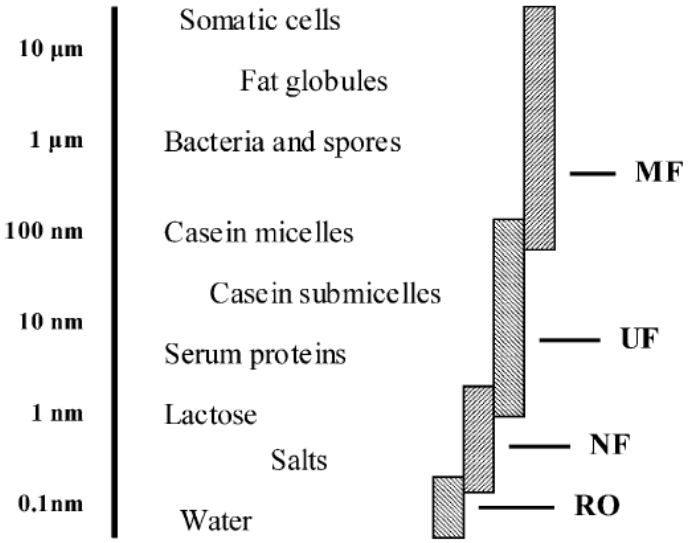


Figure 2-4: Membrane process classification based on the size of milk component. Abbreviation: MF- microfiltration, UF- ultrafiltration, NF- nanofiltration, RO- reverse osmosis (Brans et al. 2004).

Wide pore size distribution, membrane fouling and concentration polarization phenomena are the main reasons for unsuccessful application of UF for protein separation (Van Reis et al. 1997). However, a new version of UF, known as high performance tangential flow filtration (HPTFF), has been developed to overcome these limitations. HPTFF exploits a number of different strategies by selecting specific conditions such as (Van Reis et al. 1999; Van Reis et al. 1997; Zydney 1998):

- proper choice of pH and ionic strength to maximize the differences in the effective hydrodynamic volume of the different proteins
- use of electrically charged membranes to enhance the retention of charged proteins
- operation in the pressure-dependent regime to maximize the selectivity
- use of a diafiltration mode to wash impurities through the membrane

The success of HPTFF in proteins separation of similar size has been demonstrated by several researchers as demonstrated in table 2-7 (adapted from Zydney 1998). The application of HPTFF has gradually been applied to whey protein fractionation in the past 10 years. The following paragraph will summarize some related membrane based separation processes for the purpose of whey protein fractionation.

A few studies have concentrated on the optimization of membrane operating conditions in order to isolate the protein of interest from whey. Muller et al. (2003b) conducted a series of experiments by varying physico-chemical and hydrodynamic operating conditions on UF of α-Lac from whey. In the first part of the experiment, the effect of transmembrane pressure (TMP), whey concentration, ionic strength, pH and flow velocity were studied using Carbosep™ 150 kDa ceramic membrane from Orelis (St-Maurice-de-Beynost, France). Then, different types of tubular membrane, Ceram™ 150 kDa, 220 kDa and 300 kDa (Tami Industries, Nyons, France) were tested. The α-Lac purity was increased from 0.25 in initial liquid whey to 0.44 in permeate, with the yield around 53% using 300 kDa membrane.

In a study by Almecija et al. (2007), they selected a 300 kDa tubular ceramic membrane to fractionate acid whey into permeate fraction of α-Lac and β-Lac, and retentate fraction of BSA, LF and IgG. The membrane was run in a continuous diafiltration mode (4 diavolumes) at various pH, ranges from pH 3 to pH 10. Different flux was pattern was observed at different pH values. Based on the result, the purity of BSA and LF was 1.5 times higher than the original whey (2.4 and 0.9%, respectively) when operated at pH 3 and 9. For IgG, purity was improved 1.6 times from the initial value of 9.4% at pH 9. More recently, Metsamuuronen and Nystrom (2009) tested a series of Nadir® UF membrane (Celgard/Microdyn) with different hydrophobicities and different pore sizes (20, 30, 50 and 100 kDa) for enrichment of α-Lac from fresh whey. The effects of whey pH, cross flow TMP and temperature on protein transmission were studied. Using a Nadir UH030 30 kDa PES membrane operated at pH 6.4 and 40°C, 23-fold α-Lac/β-Lac ratio was achieved in permeate stream in comparison with initial whey ratio.

Table 2-7: Protein separation using high performance tangential flow filtration (adapted from Zydney 1998).

Retentate product	Permeate product	MW ratio	ΔpI	Selectivity	Membrane	Reference
BSA	Lysozyme	4.8	6.2	170	PTTK 30 kDa PSF (Millipore)	Iritani et al. 1995
BSA	Myoglobin	3.9	2.2	25	Diaflo YM30 cellulose (Amicon)	Nakatsuka and Michaels 1992
BSA	Hemoglobin	1.0	2.2	140	Omega 100kDa PES (Filtron)	Van Eijndhoven et al. 1995
BSA dimer	BSA monomer	2.0	-	32	Biomax 100kDa PES (Millipore)	Van Reis et al. 1997
IgG	BSA	2.3	2.2	50	Omega 100kDa PES (Filtron)	Saksena and Zydney 1994
Myoglobin	Cytochrome C	1.3	2.0	40	Prototype polyacrylonitrile	Yang and Tong 1997

A drastic selectivity improvement of targeted whey protein through UF membrane could also be achieved by using a charged membrane. In a study by Cowan and Ritchie (2007), PES membrane (100 kDa, Millipore) was modified with negatively sulfonated group. The finding showed that α-Lac selectivity was 5 times better as compared to unmodified membrane at pH 7.2 based on single protein transmission experiment. Similar improvement was also demonstrated in a study by Lucas et al. (1998) who used inorganic membrane (Carbosep 150 kDa, Tech-Sep) coated with positively charged polyethyleneimine. At pH 7 and low ionic strength (< 0.02 mol L^{-1}), the transmission of β-Lac was reduced to 1% while α-Lac transmission was 10%, which gave selectivity close to 10. More recently, Bhushan and Etzel (2009) modified regenerated cellulose membrane (YM30 30 kDa, Millipore) with a positively charged quaternary amine to enhance the transmission of neutral glycomacropeptide (GMP) from cheese whey at pH 3. The selectivity of GMP to other whey proteins of 13 was obtained by this charged UF membrane.

In another option, membrane filtration is combined with other unit operations in order to achieve the desired recovery for particular protein from whey solution. Xu et al. (2000), for example, used a combination of batch adsorption and ultrafiltration to recover IgG from acid whey and colostral whey. In this study, major acidic whey protein (β-Lac, α-Lac, BSA) was removed from 2 L whey by batch adsorption into Amberlite IRA93 (20% w/v) anion exchanger resin in four cycles to give a spent whey which is enriched in IgG content. The removal percentage for acidic whey protein was 95 %, 97% and 52% for α-Lac, β-Lac and BSA respectively in spent whey. The effect of pH and NaCl addition on the operation of YM100 10 kDa (Amicon) UF membrane for spent whey filtration was then studied. IgG was recovered on the retentate side of UF membrane and was subjected to diafiltration for further removal of small protein impurities. The final IgG powder recovered from acid and colostral have a 43% and 93% of IgG respectively, based on the total solid content.

Bhattacharjee et al. (2006) used two stages UF membrane (10 and 30 kDa PES, Millipore) in rotating disc membrane module followed by anion exchanger membrane chromatography (Vivapure™ Q Mini-H column) to isolate β-Lac from whey protein concentrate. The effects of stirrer speed, membrane disc rotation, TMP

and solution pH on permeate flux and rejection were investigated in UF stage. The researchers suggested that the pH of whey should be kept in the range where β-Lac exists as a monomer (pH below 3) in order to obtain a highest separation between α-Lac and β-Lac in UF module. The purity of final β-Lac products achieved after membrane chromatography step is 87.6%. Konrad et al. (2008) combined UF process with trypsin hydrolysis of β-Lac in order to recover adequate purity of α-Lac from sweet whey. The purity of α-Lac in the permeate fraction of Omega 100 kDa PES (Filtron), operated at optimal condition of 45°C, 2 bar TMP and pH 6.7, was only 36%, which was 2.6 times higher than its initial value in whey. In order to increase the purity of α-Lac, this permeate fraction was further treated with trypsin hydrolysis to degrade all β-Lac found in the solution. The hydrolysate was subjected to second UF and diafiltration using a 10 kDa membrane to give the final α-Lac purity about 90–95%.

Based on the various studies described above, membrane filtration has great potential for isolating a single protein fraction from the whey. With the introduction of the high performance tangential flow filtration concept, it is believed that membrane filtration will become common in the near future especially for α-Lac recovery from whey.

2.10 Membrane chromatography for whey protein fractionation

There is a potential for membrane chromatography to be used as a large scale whey protein fractionation for several reasons. The similarities between membrane chromatography module and the existing ultrafiltration membrane in terms of processing configurations could be one of the benefits for installing membrane chromatography without the need of expensive changes in dairy plant (Etzel 1995). In view of the large volumes of whey that are produced every day in milk processing plants, a high throughput chromatographic system such as membrane chromatography has a significant potential for improving process efficiency (Splitt et al. 1996). In addition, membrane chromatography is not a volume dependent, rather it depends mostly on the capacity of the adsorbent in the membrane.

Table 2-8 shows the application of membrane chromatography for whey protein fractionation. Splitt et al. (1996) demonstrated that the chromatographic conditions were transferable from the cellulose- to the polymer-based membrane adsorbers carrying the same functional groups for whey protein fractionation. Under the optimized binding conditions at pH 6.5 with fine-tuned gradient elution, they are able to resolve BSA, α-Lac and β-Lac in single elution peak. Freitag et al. (1996) investigated the concept of mixed mode interaction membrane chromatography to bind all whey protein in a single pass through a connected series of membrane modules. Two modules of commercial anion exchanger membrane adsorber (Sartobind MA Q15, Sartorius, Gottingen, Germany) and one module of cation exchanger membrane chromatography (Sartobind MA S15, Sartorius) were connected in series and whey passed through at pH 6. However, elution of anion and cation module was done separately because α-Lac and IgG was eluted at the same salt concentration. Sartobind MA Q15 managed to resolve a single peak of α-Lac, BSA, β-Lag A and β-Lac B, while Sartobind MA S15 gave a single peak of IgG.

Recent studies by Goodall et al. (2008) used anion exchange membrane chromatography to selectively bind β-Lac from whey. Goodall et al. (2008) observed in their flow through experiment that when the anionic membrane was saturated with whey, at some stage β-Lac could displace the other bound protein from the membrane. This can produce a flow through fraction that was depleted in β-Lac with increased concentration of α-Lac and BSA as compared to the original concentration in whey.

For basic whey protein fractionation, Chiu and Etzel (1997) measured the breakthrough curves for LP and LF in sweet whey using a Sartobind membrane adsorber and demonstrated sustained membrane adsorber performance after 12 repeated cycles of loading and elution, without cleaning between cycles. Ulber et al. (2001) used a two-step membrane process to recover LF from sweet whey. The first non-adsorptive step removed insoluble particles, such as lipids, caseins and precipitated proteins, by cross-flow filtration. The permeate stream was then fed to a commercial Sartobind cation exchange membrane adsorber. LF and LP were bound then eluted with a three-step NaCl salt gradient, yielding almost 95% LF purity. Plate et al. (2006) demonstrated that LF, LP and enzymatically prepared lactoferricin could

Table 2-8: Whey protein fractionation using membrane chromatography.

Author	Membrane type	Ligands	Protein of interest	Protein source
Goodall et al. 2008	Sartobind MA D-type and Q-type (Sartorius)	Q, DEAE	β-Lac, α-Lac, BSA	whey, single β-Lac, α-Lac and BSA, binary β-Lac and BSA
Wolman et al. 2007	PSF hollow fiber MF	Red HE-3B dye	LF	colostrum, whey
Bhattacharjee et al. 2006	Vivapure Q Mini-H (Vivasciences)	Q	β-Lac, α-Lac	permeate from two stage UF
Plate et al. 2006	Sartobind MA S15, S-type cat. # S-10k-15-25 (Sartorius)	SP	LF, LP, LFcin	whey
Ulber et al. 2001	Sartobind S-type cat. # S-10k-15-25 (Sartorius)	SP	LF	whey
Girardet et al. 1998	MemSep 1000 (Millipore)	DEAE	β-Lac, α-Lac, BSA,	whey
Chiu and Etzel 1997	Sartobind MA S120 (Sartorius)	SP	LF, LP	whey
Splitt et al. 1996	Sartobind MA Q15, MA Q100, MA D15 (Sartorius)	Q, DEAE	β-Lac, α-Lac, BSA	whey
Freitag et al. 1996	Sartobind MA Q15, MA S15 (Sartorius)	Q, SP	β-Lac, α-Lac, BSA, IgG	whey
Weinbrenner and Etzel 1994	MemSep 1010 (Millipore)	SP	α-Lac, BSA	single α-Lac, single BSA, binary α-Lac and BSA

Abbreviations: PSF - polysulfone, MF - microfiltration, Q - quaternary ammonium, SP - sulfopropyl, DEAE - diethyleaminoethyl, α-Lac - α-lactalbumin, β-Lac - β-lactoglobulin, BSA - bovine serum albumin, LF - lactoferrin, LFcin- lactoferricin, LP - lactoperoxidase, IgG - immunoglobulin.

be recovered with a Sartobind membrane adsorber at high purity and biological activity. The membrane was easily scaled up to 2 m^2, with an LF recovery of about 88%.

Contrary to most previous studies which used ion exchange, Wolman et al. (2007) used affinity membrane chromatography (triazinic dye, RED HE-3B) for LF recovery from bovine whey and colostrum. A static binding capacity of 111 mg LF mL^{-1} was obtained and in flow through experiments, the LF recovery from colostrum was about 89%. The application of membrane chromatography for whey protein fractionation was predicted to be expanded and become more important in the future years.

2.11 Other separation techniques for whey protein fractionation

Besides chromatographic and membrane based separation techniques described in the above section, there are still an abundance of other techniques that have been employed in whey protein fractionation. This section highlights some of these methods although it is not the objective to cover all of them exhaustively.

β-Lac was selectively isolated from the whey by forming a complex with chitosan (Casal et al. 2006; Montilla et al. 2007), by addition certain concentration of ammonium sulphate (Lozano et al. 2008), by precipitation of α-Lac with sodium citrate (Alomirah and Alli 2004) and by peptic hydrolysis followed with membrane filtration (Konrad et al. 2000).

β-Lac interacted reversibly with chitosan by electrostatic interaction and formed a precipitate at pH 6.2. The β-Lac can be recovered by dissolving the precipitated in 100 mM sodium acetate, pH 9 to give a recovery of 90% with a purity of 95% (Montilla et al. 2007). The isolated β-Lac can maintain its native structure and such use of non toxic chitosan would be of interest by in the industrial application. Meanwhile, Alomirah and Alli (2004) recovered a β-Lac from the supernatant of the α-Lac precipitation with sodium citrate. After several additional steps (i.e. washing, centrifuge, dialysis), single β-Lac with the purity ranging from 83-90% was

recovered. The yield of the β-Lac isolated from this process was reported in the range of 47-69%.

A study by Lozano et al. (2008) showed that by precipitation with 50% $(NH_4)_2SO_4$, β-Lac could be separated from the other whey protein. The precipitate was dissolved and separated again using 70% $(NH_4)_2SO_4$, leaving a supernatant liquid enriched in β-Lac. After dialysis, lyophilize and reconstitute in water, the final purification was done by weak cation exchange chromatography. The total yield and purity of β-Lac from 3.5 L whey was 14.32% and 95% respectively.

Konrad et al. (2000) compared four different techniques to isolate β-Lac from whey in large scale operations. These techniques were based on a peptic treatment, trichloroacetic acid precipitation, salting out procedure and selective thermal precipitation. The theoretical yield of native β-Lac after being normalized to 1 kg of whey achieved by different methods was 67.3, 44.9, 46.7 and 49.6% for the peptic treatment method, acid precipitation, salting out procedure and thermal precipitation respectively. The purity of β-Lac achieved by all methods was more than 90%. The peptic treatment technique was successfully applied to treat 10,000 L of whey without any difficulty in technical operation and product quality.

Chemical precipitation with sodium hexametaphosphate was used to recover α-Lac from whey (Alomirah and Alli 2004). The yield of α-Lac precipitation with sodium hexametaphosphate was reported about 44-89% with α-Lac purity between 86-90% (Alomirah and Alli 2004). Tolkach et al. (2005) used selective thermal precipitation to isolate native α-Lac from WPC. Before precipitation, the environmental parameters of WPC were optimized in terms of total protein, lactose and calcium content, as well as pH value. The purity of α-Lac achieved by selective thermal precipitation of optimized properties of WPC was 98% with a recovery about 75%.

Muller et al. (Muller et al. 2003a; Muller et al. 2003b) used two step processes to purify α-Lac from whey protein concentrates. In the first step, whey was filtered through 30 kDa UF with a proper operating condition to enhance the ratio of α-Lac/β-Lac in permeate stream by minimizing the passing of other whey proteins (Muller et al. 2003a). In the second step, two options were investigated, second UF

module or selective precipitation route using citric acid (Muller et al. 2003b). The precipitation route was more promising as compared to UF, with the purity of α-Lac achieved in the range of 77-99% and yield of 46-83%, depending on the permeate properties from the first step of UF. Due to the excellent properties and promising application of whey protein components, it is believed that new technique was explored from time to time to achieve a single protein fractionation in economic ways.

2.12 Conclusion

The efforts in producing single proteins from whey grow continuously from year to year. Although most new techniques are based on lab scale operation, some of them have potential for large scale operation. Expanded bed chromatography for instance, could capture directly whey protein components without requiring whey pre-treatment steps. High column backpressure is also absent in this operation mode compared to normal packed bed column chromatography. However, it is complex and consumes large amounts of buffer. In conventional membrane separations based on size, high performance tangential flow filtration has good potential for isolating small proteins such as α-Lac. With proper manipulation of the buffer system and membrane properties, α-Lac can be selectively permeated through the membrane while leaving other proteins in the retentate stream. However, the permeate is typically dilute and the need to manipulate the feed material conditions is a drawback compared with processing milk or whey directly.

Membrane chromatography, which combines the principles of chromatography and membrane filtration, seems to have potential for whey protein fractionation. Membrane chromatography is not volume dependent but depends mostly on the capacity of the adsorbent in the membrane. It also can be operated at high throughput without affecting its performance. However, the normal routine for preparation of chromatographic membranes seems to be very complicated and involves many chemical steps. The concept of MMM preparation is interesting and feasible for large scale membrane manufacturing. However, until recently no one has applied MMM chromatography to whey protein separations. Therefore, the objectives of the current

study were to prepare various types of MMMs and to test their feasibility for applications in whey protein fractionation.

3 General materials and methods

3.1 Materials

EVAL (a random copolymer of ethylene and vinyl alcohol) with an average ethylene content of 44 mol% was purchased from Sigma (St. Louis, MO, USA) and used without chemical modification to cast membranes. Dimethysulfoxide (DMSO) (Fluka, Steinheim, Germany) was employed as the EVAL solvent and 1-octanol (Fluka) as a non-solvent additive in casting solutions. Adsorptive resins MMM include Lewatit MP500 (Lanxess) for anionic MMM, SP Sepharose Fast Flow (GE Healthcare Technologies) for cationic MMM and Phenyl Sepharose Low Substitute (GE Healthcare) for hydrophobic interaction MMM.

β-Lac, α-Lac, BSA, LZY and cytochrome C were purchased from Sigma (St. Louis) and used without further purification. LF of known purity was sourced from Tatua Co-operative Dairy Company Ltd, Morrinsville, New Zealand.

Sodium phosphate binding buffers were prepared from sodium phosphate dibasic heptahydrate (Sigma), sodium dihydrogen orthophosphate 1-hydrate (BDH Chemicals, Poole, England) and NaCl (BDH Chemicals). For sodium acetate buffer, sodium acetate (Merck, Darmstadt, Germany) and acetic acid (J. T. Baker, Philipsburg, NJ, USA) were used. $(NH_4)_2SO_4$ for hydrophobic interaction buffer was purchased from BDH Chemicals. All buffer solutions were prepared using deionized (DI) water.

3.2 Preparation of mixed matrix membrane

Figure 3-1 showed the steps involved in preparing a MMM during this study. A homogenous polymer solution, consisting of 15 wt% EVAL polymer and 15 wt% 1-octanol in DMSO was prepared by continuous stirring at about 60°C for several hours until all EVAL pellets were completely dissolved. The adsorbent resin to be incorporated into the polymer solution was firstly ground into a smaller size. The resin was ground using an ultra centrifugal mill (Retsch ZM100, Haan, Germany)

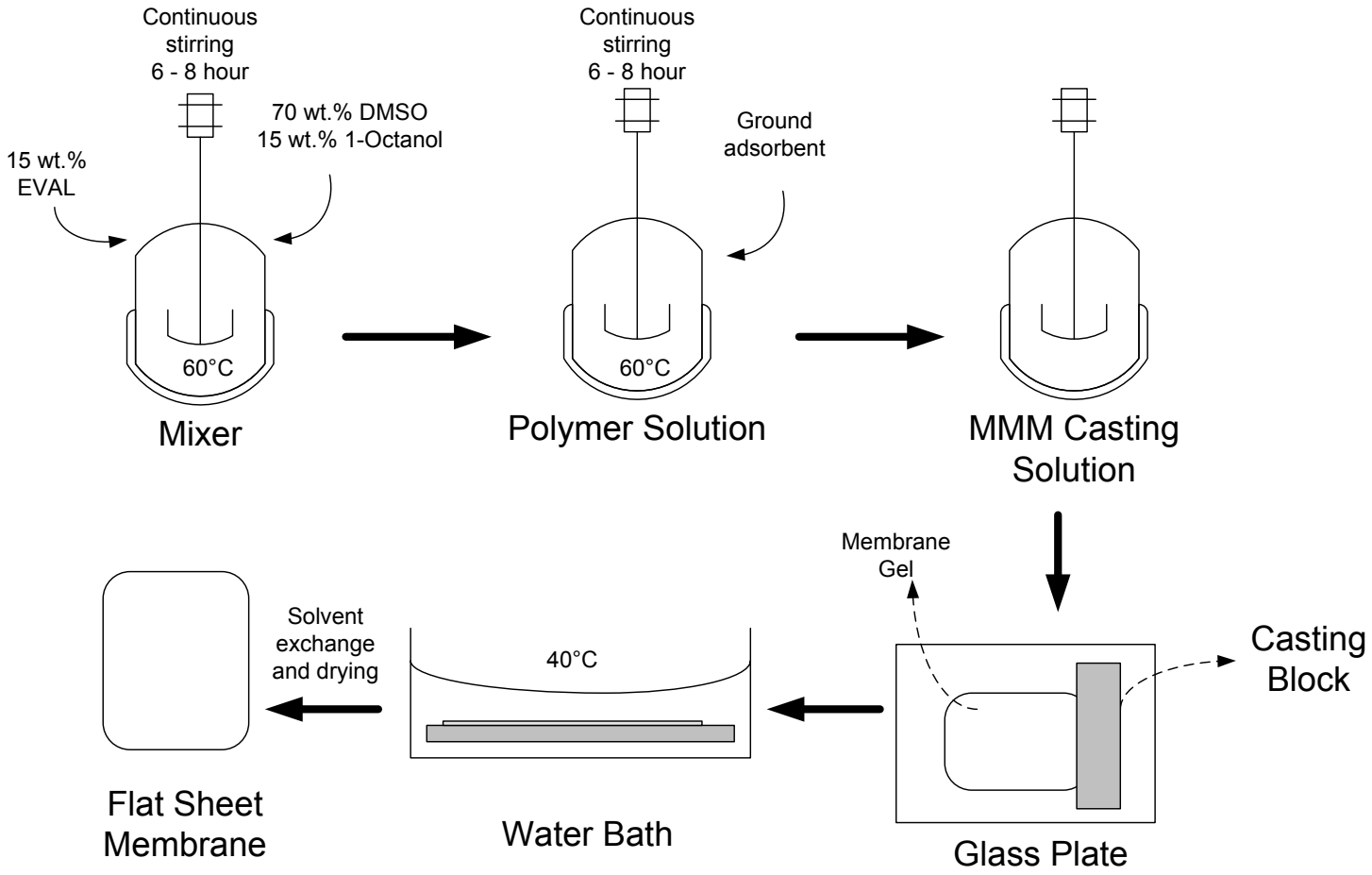


Figure 3-1: Preparation of mixed matrix membrane chromatography.

and screened to obtain a particle fraction that passed through at least 38 µm stainless steel mesh (or otherwise size mentioned). Ground resin was added to the prepared polymer solution at certain weight fraction (relative to the EVAL content in the polymer solution) and this mixture was stirred until a homogeneous casting slurry was obtained.

A conventional casting method was used for making flat sheet mixed matrix membranes. The casting solution was poured onto a glass plate support and then spread to form a thin film using a stainless steel block (figure 3.2) with a 400 µm recess milled into the bottom surface. Immediately after casting, the glass plate with the film on the surface (now gelled through brief exposure to the atmosphere) was carefully immersed for coagulation in a water bath at 40°C until the membrane was completely solidified and detached from the glass. The resulting mixed matrix membrane was washed with water several times and left in the water bath overnight to completely remove traces of solvent from the membrane structure. The wet MMM was then freeze-dried to remove water without affecting the structure of the membrane.

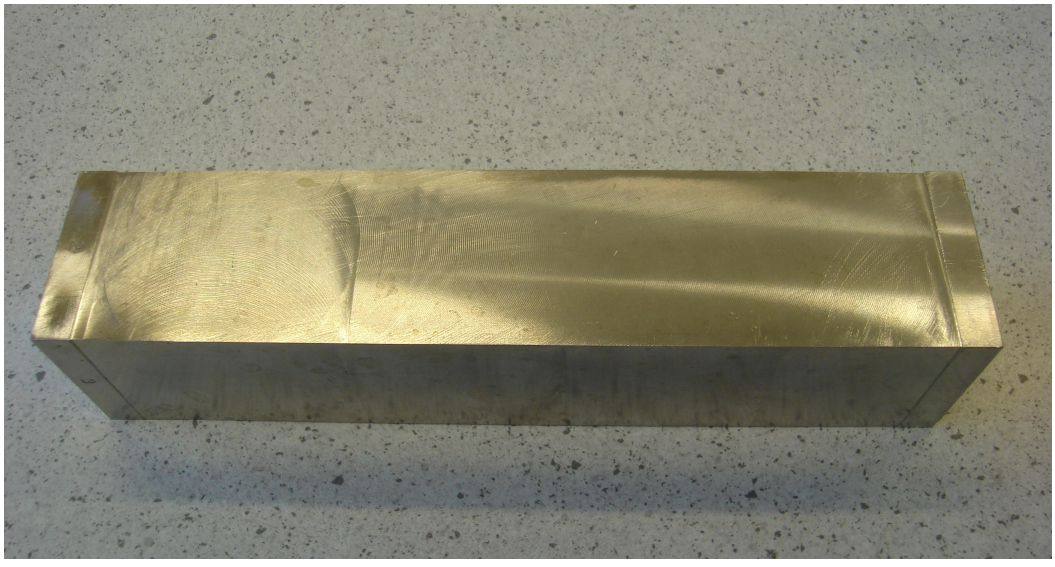


Figure 3.2: Membrane casting block made from stainless steel 316. The length of the block is about 220 mm with 45 mm equal width and height. Each edge of the block was milled to have different recess thicknesses of 200, 300, 400 and 500 µm.

3.3 Preparation of whey

Skim (0.05% fat) milk was purchased from a retail store and heated to 40 °C in a water bath. Casein was precipitated by adjusting the pH between 4.6-4.8 with 0.5 M HCl under stirring. The precipitated casein was discarded and the whey supernatant was centrifuged at 17,902 *g* at 4 °C for 20 min using an Eppendorf Centrifuge Model 5810R. Final whey filtration was achieved with a 0.45 μm membrane filter. The whey was adjusted to the desired pH using 0.5 M NaOH.

3.4 Membrane porosity

Membrane porosity was measured according to previously published methods (Avramescu et al., 2003; Saiful et al., 2006), with porosity, ε (%), defined as:

$$\varepsilon = \frac{V_{wet} - V_{dry}}{V_{wet}} \times 100\% \qquad (3\text{-}1)$$

where V_{dry} and V_{wet} are, respectively, the volume of the dry membrane and the volume of the swollen membrane after 24 h of immersion in a water bath at room temperature. The volume of dry membrane was calculated as the ratio between the weight of dried membrane and the polymer density. Before weighing the wet membrane, surface water was removed by lightly patting the membrane with filter paper. Average values were obtained from at least three different samples.

3.5 Static binding capacity

A known mass (dry basis) of adsorbent in different format (i.e. intact or ground resin, membrane) was equilibrated in binding buffer, 20 mM sodium phosphate pH 6.0 for at least 3 h. Pre-equilibrated adsorbent was then incubated with a series of initial protein concentration in a small Eppendorf tubes. Adsorbent and liquid in these tubes were gently mixed by inversion throughout binding for at least 12 h at room temperature. The binding capacity of each adsorbent was determined by the

difference between initial and the final total solution protein content per mass of adsorbent used. All experiments were carried out at least in triplicate.

Static (equilibrium) binding capacity data was tested to fit using either Langmuir or Freundlich adsorption isotherm using a least-square regression method. Langmuir isotherm is given in Equation 3-2 and Freundlich isotherm is given in Equation 3-3. In these equations, q and q_m are the equilibrium and maximum protein binding capacities in mg protein bound g^{-1} adsorbent respectively, c is the equilibrium protein concentration in mg mL^{-1}, K_d is the Langmuir dissociation constant in mg mL^{-1} and K and n are the Freundlich constants.

$$q = \frac{q_m c}{K_d + c} \tag{3-2}$$

$$q = Kc^n \tag{3-3}$$

3.6 Dynamic binding capacity

Sheets of MMM were cut into circles of diameter 44 mm to fit into a 47 mm diameter polypropylene filter holder (GE Osmonics Labstore, Minnetonka, MN) for dynamic binding experiments by dead-end filtration. A single membrane (volume 0.304 mL) was used in all experiments. The filter holder was connected to an AKTAexplorer™ 100 (GE Healthcare Technologies) liquid chromatography system controlled by Unicorn™ software (GE Healthcare Technologies, Uppsala, Sweden). The membrane was equilibrated with a binding buffer before each protein or whey solution was continuously injected into the module. The permeate was collected using a fraction collector and the concentration in each fraction was assayed. If the same membrane was used for repeated experiment, the membrane was cleaned with 0.5 M NaOH solution (30 min, 1 mL min^{-1} permeate flow rate) and flushed with water until the permeate pH was neutral.

The dynamic binding capacity was determined at 10% breakthrough using Equation 3-4, where q_{DBC} is the dynamic binding capacity, V_{BT} is the permeate volume at 10% breakthrough, c_F is the feed protein concentration and m is dry mass of the MMM.

$$q_{DBC} = \frac{V_{BT} \times c_F}{m} \tag{3-4}$$

3.7 Single protein assay

3.7.1 UV-visible spectrophotometer

Most of the single protein concentrations were assayed by measuring absorbance of solution at 280 nm using an UV-Visible spectrophotometer (Model Ultraspec 2100 Pro, Amersham Biosciences, England). An absorbance-concentration standard curve was developed from six standard concentrations from 0 to 1 mg mL^{-1}. Samples were diluted with reference buffer to within the absorbance range of the standard curve.

3.7.2 Size exclusion chromatography

Single protein concentrations of β-Lac and α-Lac for determined a static binding capacity of anion exchanger MMM were measured by size exclusion chromatography using a Superdex200™ HR 30/10 column (GE Healthcare Technologies) (length 300 mm, diameter 10 mm) attached to an AKTAexplorer 10 liquid chromatography system controlled by Unicorn 4.0 software. Protein samples (40 μL) were injected into the column by an autosampler and eluted isocratically with one column volume of phosphate buffered saline (pH 7.4) at 0.5 mL min^{-1}. The absorbance of the system was continuously monitored at 280 nm and the protein concentrations were determined by comparing the peak areas of unknown samples with those of a set of standard samples.

3.8 Whey protein assay

Whey protein components were assayed using a reverse phase chromatography (RPC) column following the established method by Elgar et al. (Palmano and Elgar 2002; Elgar et al. 2000). The assay was run on an AKTAexplorer 10 liquid chromatography system controlled by Unicorn 4.0 software with samples manually injected through a sample injection loop. A 1 mL Resource™ RPC column (GE Healthcare Technologies) was used with 0.1% v/v trifluoroacetic acid (TFA) (Sigma) in DI water as buffer A and 0.09% v/v TFA, 90% acetonitrile (J. T. Baker) in DI water as buffer B. The column was equilibrated with five CV's of 20% buffer B before a 500 µL sample was injected manually. A series of linear gradients were then applied as follows: 0-1 CV, 20% B; 1-6 CV, 20-40% B; 6-16 CV, 40-45% B; 16-19 CV, 45-50% B; 19-20 CV, 50% B; 20-23 CV, 50-70% B; 23-24 CV, 70-100% B. The column was then held for 1 CV at 100% B, followed by a 2 CV linear gradient to 20% B, then held for 3 CV. Detection was by absorbance at 214 nm and a flow rate of 2 mL min^{-1} was used. The standard curve of peak area versus concentration was developed using a dilution series from a mixture of pure proteins corresponding to their composition in whey. Figure 3-3 shows an example of whey chromatogram assayed by this technique.

3.9 Gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was run using a NuPAGE® Novex® 4-12% Bis-Tris Midi Gel from Invitrogen (Carlsbad, CA, USA) under non-reducing or reducing conditions. Protein samples were mixed with NuPAGE LDS 4X (Invitrogen) sample buffer and/or NuPAGE Reducing Agents 10X (Invitrogen), and heated at 80°C for about 10 minutes. The gel was mounted into a XCell4 SureLock™ Midi-Cell (Invitrogen), filled with NuPAGE MES SDS (Invitrogen) running buffer and connected to PowerPac™ HC (BioRad, Hercules, CA, USA) power supply. 5 µL of Novex Sharp Pre-Stained (Invitrogen) protein standard (or in some experiment using BioRad Precision Plus Protein Pre-Stained Standards), was used as a protein marker and 20 µL of sample was loaded in each well. The gel was run for 40 minutes at constant voltage of 200 V. Proteins were stained with Coomassie Brilliant Blue R 250 (Sigma), 0.125% (w/v) in 10% acetic

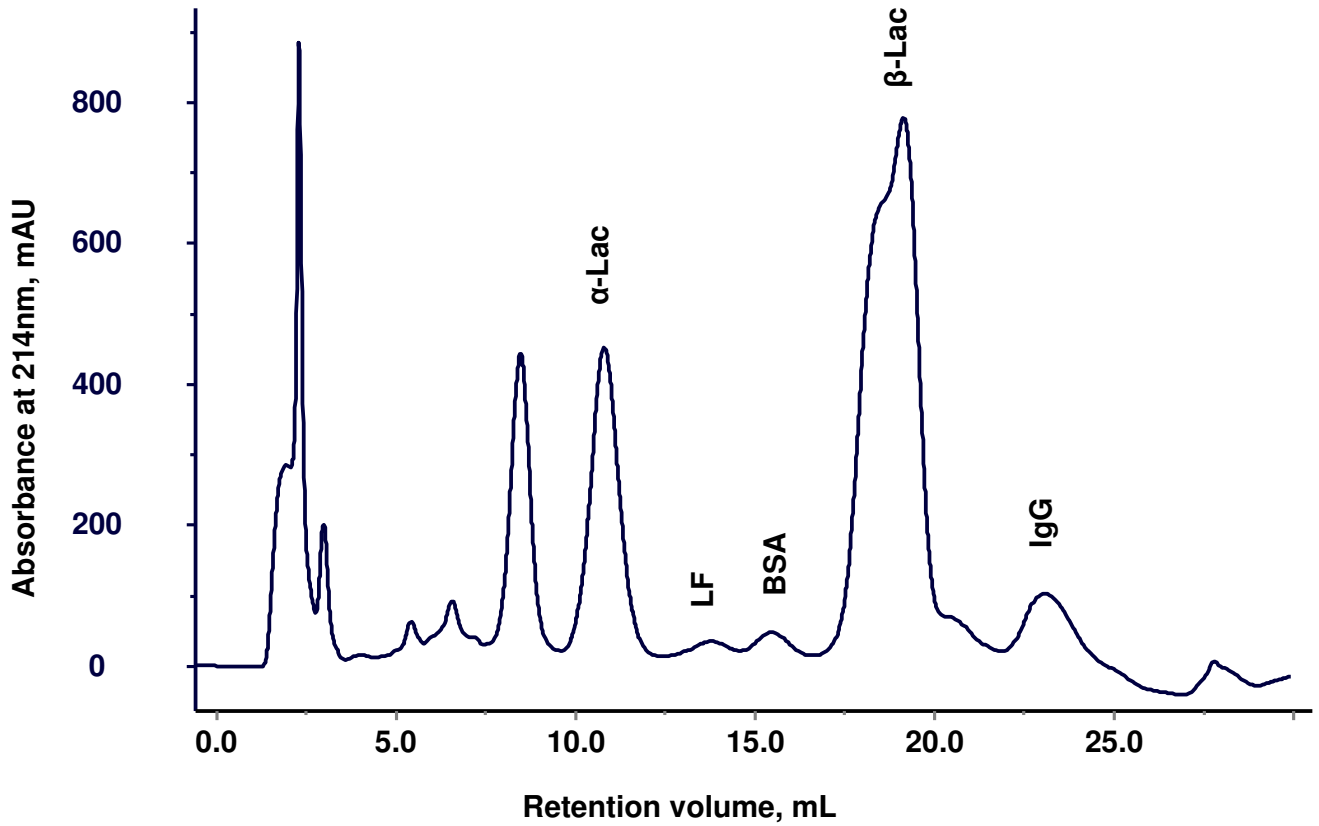


Figure 3-3: Chromatogram for whey assayed using Resource RPC 1 mL reverse phase chromatography. Abbreviation: α-Lac- α- lactalbumin, LF - lactoferrin, BSA - bovine serum albumin, β-Lac - β-lactoglobulin, IgG - immunoglobulin.

acid and 40% methanol. Destaining was carried out in a solution of 10% acetic acid and 20% methanol in water. Typical SDS-PAGE gels for whey protein under reducing and non-reducing conditions are shown in figure 3-4.

3.10 AKTA cross-flow system

Cross-flow experiments were run using an AKTAcrossflow™ (GE Healthcare Life Sciences) tangential flow filtration system. The filtration system was fully automated and controlled by Unicorn 5.11 software.

The main system components were a 350 mL reservoir tank, the membrane module, a transfer valve (8 lines), retentate valve (3 lines) and a permeate valve (4 lines) (figure 3-5). All pressures in feed, retentate and permeate lines were recorded continuously by the system. On the permeate side, conductivity, pH and UV absorbance were continuously monitored. The instrument could be run under either a constant flux or a constant TMP mode. A custom plate-and-frame module was fabricated to connect to the cross-flow instrument (figure 3-6). The module was rectangular, with dimensions 10.5 cm × 15.5 cm. This module could house several layers of membrane, each with an effective active membrane area of 50 cm^2.

Cross-flow experiments were conducted for LF recovery from whey using cation exchange MMM. 20 mM sodium phosphate pH 6 was used as a binding buffer and 1 M sodium chloride was added to binding buffer as an elution buffer in cross-flow experiment. The feed flow rate of 50 mL min^{-1} and permeate flux of 100 L m^{-2} h^{-1} (LMH) was used throughout the experiment, except in whey loading step where flux of 50 LMH was used.

The membrane was equilibrated with binding buffer until the cumulative permeate volume reached to a value of 300 mL. During loading, 150 mL of whey was circulated past the membrane and both retentate and permeate streams were recycled back into the feed reservoir. Whey loading was completed when the cumulative permeate volume reached 300 mL. After the loading step, a liquid in retentate side

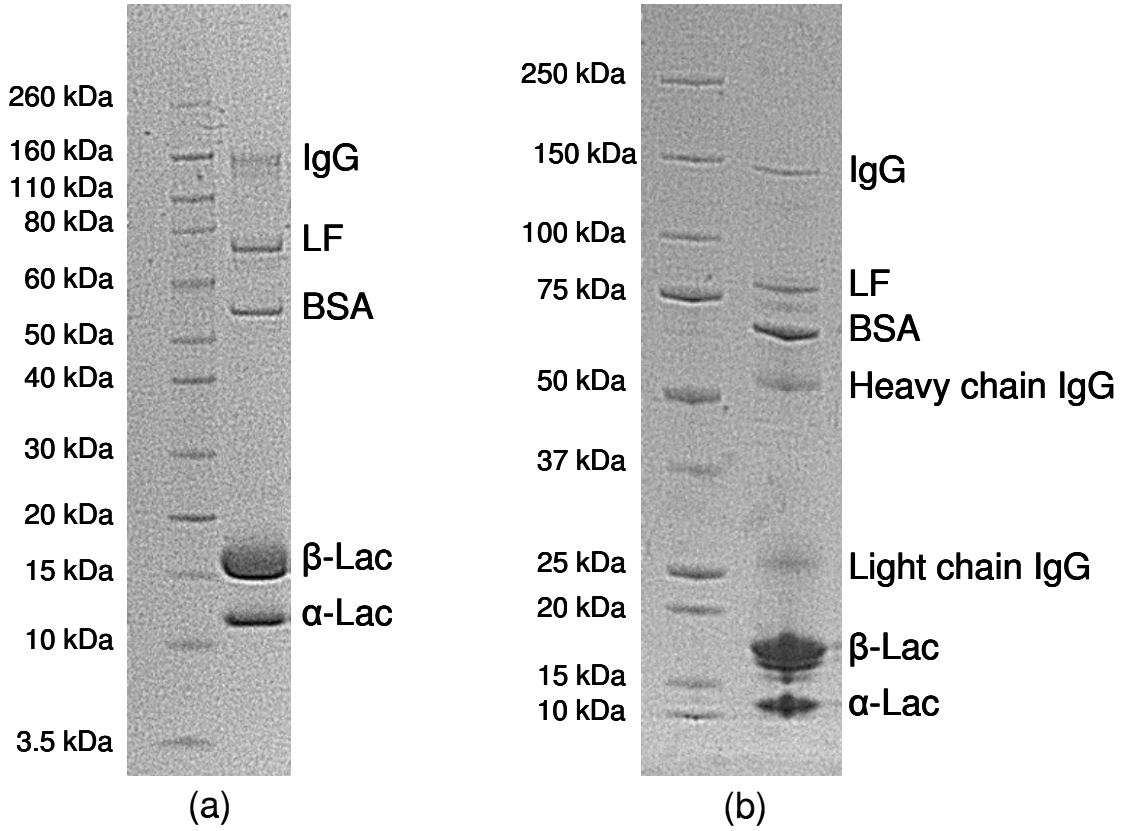


Figure 3-4: Typical SDS-PAGE for whey protein run under (a) non-reducing and (b) reducing condition.

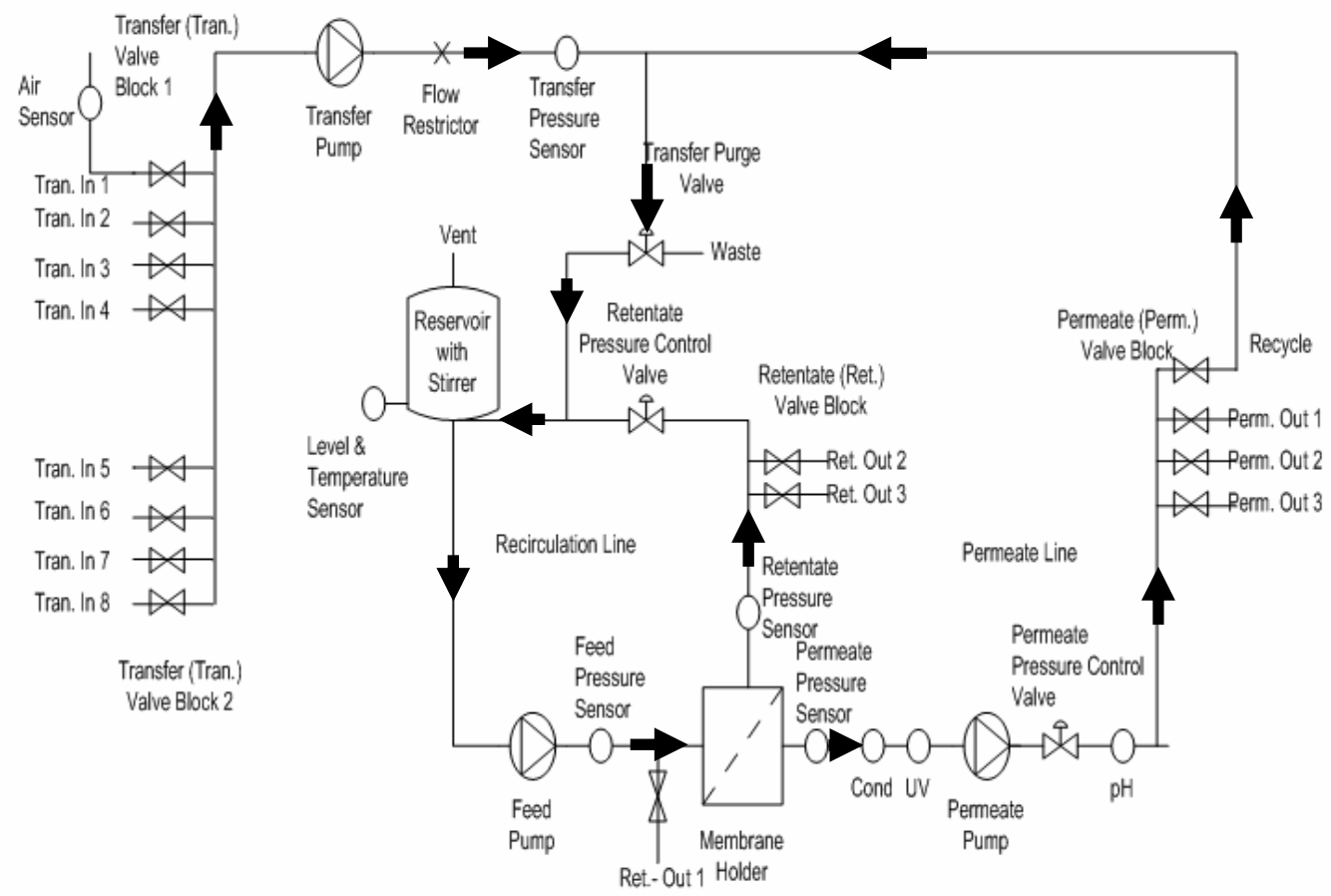


Figure 3-5: AKTAcrossflow™ tangential flow filtration system.

was drained and flushed several times with binding buffer (about 1000 mL). Then, 200 mL of fresh binding buffer was transferred to the reservoir for a washing step.

The buffer was fed into the membrane until the cumulative permeate volume reached 150 mL. The remaining fluid in the reservoir after the washing step was drained for the next step. The reservoir was filled with 120 mL of elution buffer and fed into the membrane until the permeate volume of 90 mL. All the retentate and permeate fractions in each steps were collected for the analysis. Fresh membranes were used for each part of cross-flow experiment and triplicate experiments were conducted in each experiment.

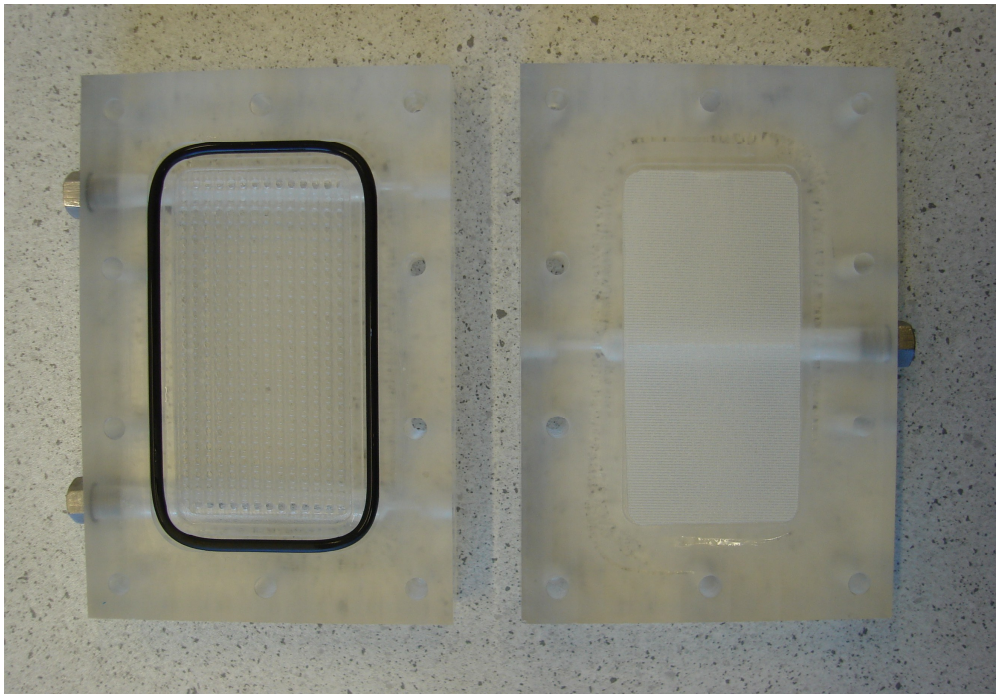


Figure 3-6: Plate-and-frame module used in AKTAcrossflow system which is made from perspex. Each part of the module has a dimension about 155 mm × 105 mm with 23 mm thickness. The effective membrane exposure dimension is about 100 mm × 50 mm which give an effective membrane area of 50 cm^2.

4 Anion exchange mixed matrix membrane chromatography for β-lactoglobulin fractionation from whey

4.1 Introduction

In this chapter, the development of anion exchange MMM chromatography for acidic whey protein fractionation is described. The MMM chromatography was prepared using EVAL polymer and Lewatit MP500 anion exchanger resin to form a flat sheet membrane. The MMM was characterized in terms of structure and its static and dynamic binding capacities were measured. The anionic MMM shows a selective binding to β-Lac in whey with a binding preference order of β-Lac > BSA > α-Lac. The β-Lac content of bovine milk (El-Agamy 2007; Fox and McSweeney 1998) is much higher than in human milk and this has been identified as a potential source of allergic reactions to infant formulae seen in some children (El-Agamy 2007; Monaci et al. 2006; Suutari et al. 2006). Selective removal of β-Lac may therefore be of interest in the commercial application of this anionic MMM chromatography.

4.2 Materials and methods

4.2.1 Anion exchanger resin

Lewatit anion exchanger, MonoPlus MP500 resin was selected as a resin to be incorporated in membrane matrix. This resin was a gift from Lanxess (Leverkusen, Germany) through their local supplier (Ashland Drew, New Zealand Limited). MP500 resin is a macroporous, strong anion exchanger made from a divinylbenzene/styrene base matrix with a quaternary amine functional group. The mean diameter of the resin particles as reported by the supplier was approximately 0.62 mm, the average pore size was 43 nm, porosity was 0.2 mL g^{-1}, surface area 20 $m^2 g^{-1}$ and the pKa of the resin was 9-9.5.

4.2.2 Preparation of mixed matrix membranes

Ground MP500 resin particles, which have a particle size smaller than 43 µm, were added to the membrane polymer solution at 50% weight fraction relative to the EVAL content in the polymer solution. This mixture was stirred until a homogenous casting slurry was obtained and was cast into a flat sheet membrane as described in detail in section 3.2. The porosity of the membrane was measured according to the protocol in section 3.4.

4.2.3 Preparation of whey

Whey was prepared from skim milk by acid precipitation of casein at pH 4.8 as described in section 3.3. The final pH of whey after a series of filtration was adjusted to pH 6.0. The concentrations of the major protein components in whey were determined using reverse phase chromatography as described in section 3.8. Table 4-1 shows the major acidic protein composition of the whey prepared by this method.

Table 4-1: Composition of β-lactoglobulin, α-lactalbumin and bovine serum albumin in whey prepared in this study. Data shown is based on average values ± one standard deviation (n=3).

Protein	Concentration, mg mL^{-1}
β-Lactoglobulin	3.716 ± 0.715
α-Lactalbumin	1.030 ± 0.141
Bovine serum albumin	0.102 ± 0.022

4.2.4 Static binding capacity

Static binding capacities of the MMM as well as intact and ground MP500 resin were determined for both α-Lac and β-Lac pure protein solutions. A known mass of each adsorbent, typically 10 mg of intact resin and ground resin, and about 30 mg of MMM (a single, rectangular piece of membrane, 12 mm × 22 mm, volume 5.3×10^{-2} mL), was equilibrated for 3 h in binding buffer at the optimum binding pH (6.0) and then incubated with six initial protein concentrations from 0.25 to 8 mg mL^{-1} protein overnight. Samples were mixed gently by inversion throughout binding. The binding capacity of each adsorbent was determined by difference between initial and final

total solution protein content per mass of adsorbent used (dry basis). The equilibrium protein concentrations were assayed using a size exclusion chromatography method (section 3.7.2). All experiments were carried out at least in triplicate. Several experiments were conducted using protein solutions with salt concentrations that gave a conductivity equal to that of whey. Binding capacities were also determined with whey by the same methods.

The effect of pH on binding was also determined. Sodium phosphate (20 mM) buffers at seven pH values (pH 4.0-8.0) were prepared and the static (equilibrium) binding capacity of the intact MP500 resin was measured. A predetermined amount of resin was measured accurately and equilibrated for 3 h in binding buffer, then incubated overnight in 3 mg mL^{-1} β-Lac in binding buffer at each pH before determining the equilibrium binding capacity as described above.

4.2.5 Dynamic binding capacity

The dynamic binding capacity of MMM for β-Lac was measured at different flow rates from 1 to 5 mL min^{-1} according to the method described in section 3.6. The binding capacity for BSA was also measured to compare it with published values for other adsorptive membranes. Using a similar method to that of Goodall et al. (2008), 1 mg mL^{-1} BSA solution was loaded on to the pre-equilibrated membrane and permeate fractions were collected and monitored for UV absorbance. Protein loading was stopped when the absorbance of the permeate reached the same absorbance as the feed solution. The membrane was then washed with binding buffer until absorbance of the permeate fell to the original baseline. The bound BSA was determined by the mass difference between the feed protein and the protein collected in the permeate fractions.

4.2.6 Batch fractionation of whey

Batch fractionation of whey was tested using small pieces of membrane of known weight. Five randomly selected samples of MMM were equilibrated for 3 h in 20 mM sodium phosphate at pH 6.0 and then incubated in whey at pH 6.0 overnight at room temperature. The membrane was removed from the whey solution and washed

with equilibration buffer for 30 min. It was then incubated overnight in an elution buffer of 1 M NaCl in 20 mM sodium phosphate, pH 6.0, to recover the adsorbed protein from the membrane.

Another experiment was conducted to find the binding preference of major acidic protein in whey to anionic MMM. Two sets of whey solution were used. The first set was whey prepared with a serial dilution of concentration using the binding buffer. The second set was a simulated whey solution consisting of the major whey acidic proteins β-Lac, BSA and α-Lac at a similar concentration ratio to whey but with the concentration of β-Lac undergoing a serial dilution while the concentrations of BSA and α-Lac kept constant at 0.15 mg mL^{-1} and 1.2 mg mL^{-1}, respectively. The membrane was first equilibrated for 6 h in binding buffer. After drying by lightly padding the membrane with adsorptive tissue, the membrane was incubated with 1 mL of whey solution overnight at room temperature (20°C).

4.2.7 Single and mixture protein assay

Single proteins were assayed using a Superdex200™ HR30/10 column (GE Healthcare Technologies) as described in section 3.7.2. For whey protein components, a 1 mL Resource™ (GE Healthcare Technologies) reverse phase chromatography column was used with the detailed protocol described in section 3.8.

4.3 Results and discussion

4.3.1 Structure of mixed matrix membranes

Membrane structure can have a significant impact on membrane performance, such as adsorption capacity and adsorption rate, especially when substances of different sizes are to be separated (Liu and Bai 2006). Sponge-like and macrovoid-free structures, with highly interconnected and open pores are desirable for adsorptive membranes because they provide large internal surface areas for adsorption and uniform flow across the membranes to ensure process efficiency (Avramescu et al. 2002; Liu and Bai 2006). The presence of macrovoids should be avoided because these cause weak spots in the membrane, leading to mechanical failure under

pressure. Furthermore, macrovoids create channels and lead to lower dynamic adsorption capacities, due to by-passing of part of the adsorptive area (Avramescu et al. 2003b).

Figure 4-1 shows the structure of the base (EVAL) membrane and the corresponding MMM by scanning electron microscope (SEM). Although the EVAL membrane has the desired open interconnected pore structure from the bottom to the top surface, there are minor macrovoids evident near the top of the membrane surface. However, these macrovoids disappeared after incorporating MP500 resin particles to form the MMM. According to Avramescu et al. (2003b) the solid particles can act as nuclei in casting solutions, limiting the growth of macrovoids. (Large holes in the MMM seen in figure 4-1 are likely due to removal of resin particles during the membrane fracture process after freezing in liquid nitrogen for SEM analysis.) The top and bottom surfaces of the MMM do not contain resin particles so retain the same permeable areas as the base membrane. Thus, MMMs are expected to have similar pressure drops to other membrane chromatography systems.

Although it would be unwise to extrapolate results, for a single membrane thickness (200 µm) we observed (data not shown) backpressures of less than 1 bar at pure water permeate flow rates up to 20 mL min^{-1}, corresponding to a flux rate of 788 L m^{-2} h^{-1}(LMH), which is well in excess of normal membrane flux rates (less than 100 LMH).

Resin grinding allows the resin particles to bind tightly within the MMM structure and improves the homogeneity of distribution of resin particles throughout the membrane matrix. A potential disadvantage of the MMM approach to creating adsorptive membranes, compared to chemically modified membranes, is that the embedded resin particles within the membrane present a diffusive path length that might slow down dynamic binding. However, resin particle size reduction by grinding also increases the interfacial surface area between permeate and resin and decreases the diffusion path length to the internal ion exchange sites, both of which improve the dynamic ion exchange capacity. Figure 4-1 shows that the resin particles are tightly held within the membrane matrix, although the uniformity of the particle size could perhaps be improved. A narrow size distribution of resin particles should

improve membrane structure and minimize batch-to-batch variations in membranes during manufacture.

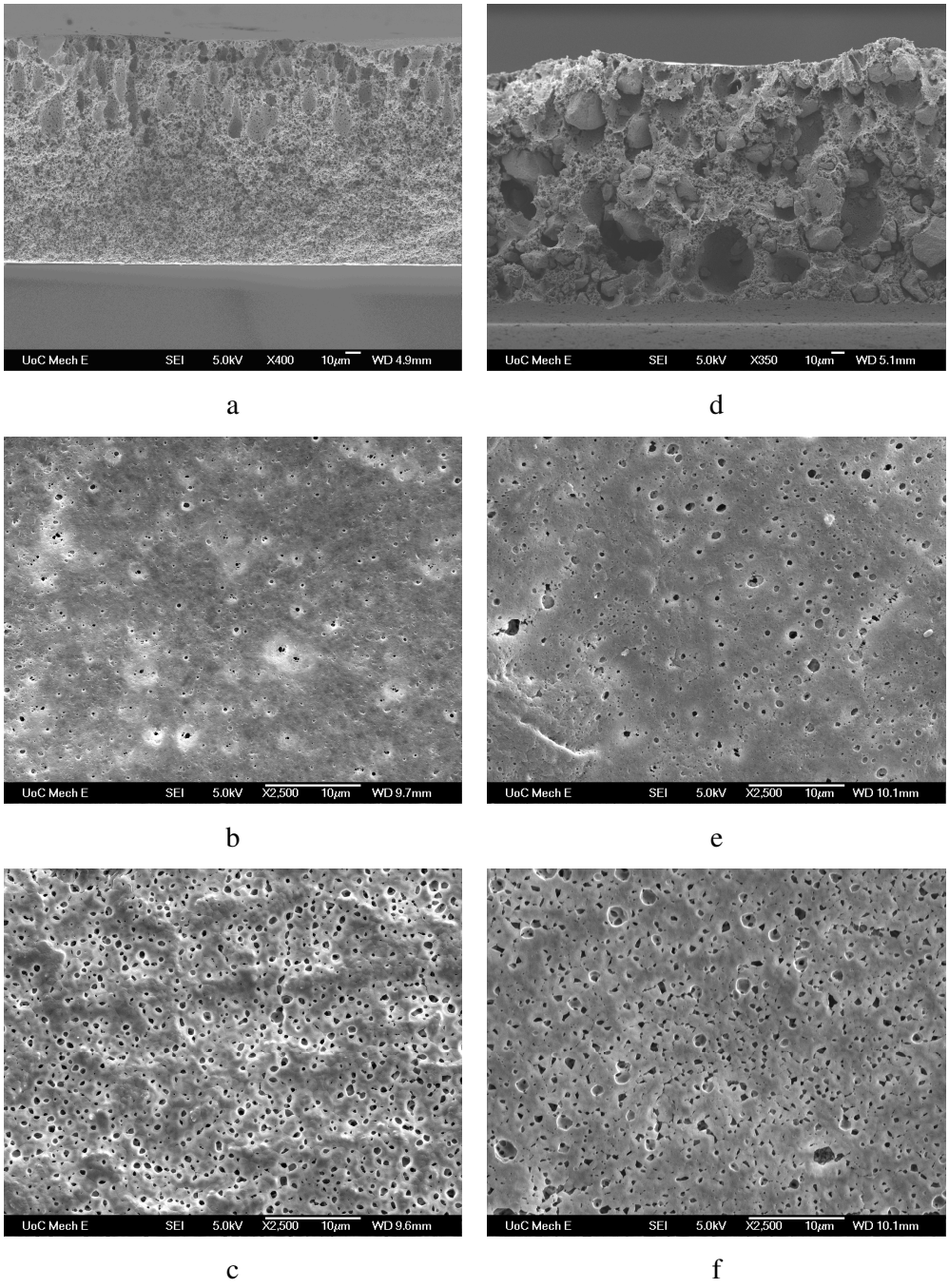


Figure 4-1: Structure of EVAL and mixed matrix membranes. (a) EVAL membrane cross section; (b) EVAL membrane top surface; (c) EVAL membrane bottom surface; (d) MMM cross section; (e) MMM top surface; (f) MMM bottom surface.

The porosity of the membrane was calculated to be approximately 74%, which is quite similar to membranes prepared by Wessling's group (Avramescu et al. 2003a; Saiful et al. 2006). The thickness of the membrane after freeze drying was measured by a micrometer to be approximately 200 μm.

4.3.2 Effects of pH on binding capacity of MP500 resin

Figure 4-2 shows the binding capacity of intact MP500 resin for β-Lac at different pH values. The maximum binding capacity was achieved around pH 5-6 which is near the pI of β-Lac (pH 5.35-5.49 (Fee and Chand 2006)). Intuitively, one would expect the binding to increase as the pH of binding deviates upwards from the pI of the protein, as the protein would be more highly charged at higher pH values. However, several authors have reported similar results where the highest binding capacity occurs near the pI of the protein (Saiful et al. 2006; Yamamoto and Ishihara 1999) and it is recognized now that ion exchange properties of proteins cannot be fully explained by the net charge concept alone (Kacar and Arica 2001). Changes of buffer pH can alter the charge distribution, charged group in the contact regions, conformational structure, and zeta potential of the surface of proteins. All of these effects will influence the protein binding to the adsorbent. According to Norde and Lyklema (1991), at the pI of protein, a compact structure is favored, due to the London–van der Waals interactions, which can allow the protein to pack more tightly on the surface of the adsorbent. One would not expect β-Lac to bind at pH values far below its pI and the low binding observed at pH 4.0 is likely due to small hydrophobic interactions or weak electrostatic interactions due to uneven charge distributions on the protein surface.

4.3.3 Binding capacity of MP500 resin

The binding capacity of intact and ground MP500 resin is shown in figure 4-3 for β-Lac and α-Lac. The data was fitted to a Freundlich adsorption isotherm (Equation 3-3) using a least-square regression method (table 4-2). A Langmuir isotherm did not provide a good fit to the data, contrary to previous work (Avramescu et al. 2003a; Avramescu et al. 2003b; Lin et al. 2001; Saiful et al. 2006). The Freundlich constants for different forms of adsorbent are shown in table 4-2.

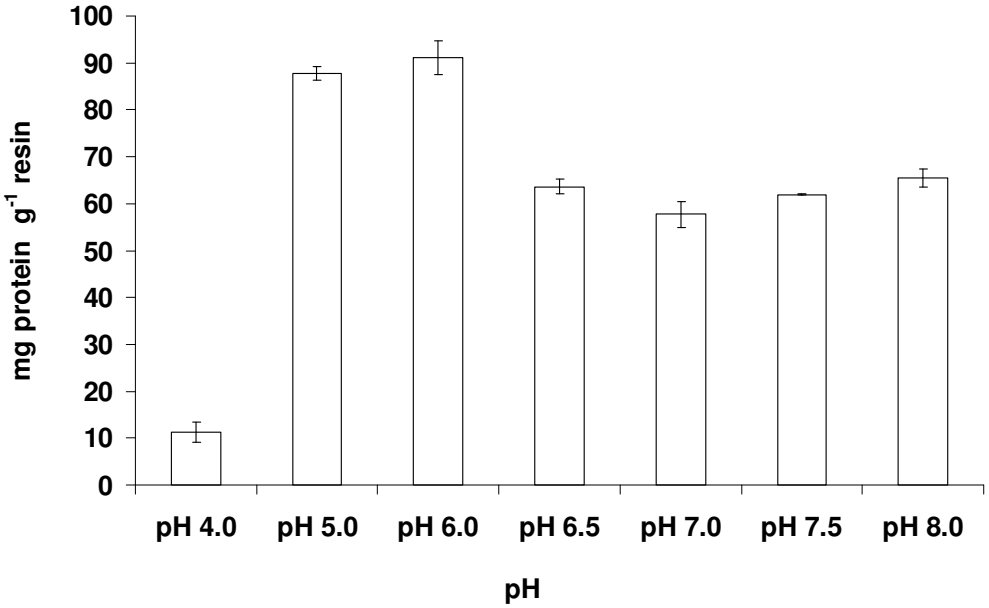


Figure 4-2: Binding capacity of intact MP500 Lewatit anion exchange resin for β-Lac at various pH values. Error bars are ± one standard deviation (n=3).

Table 4-2: Freundlich isotherm constants (Equation 3-3) for different types of adsorbents in static binding experiments.

	β-Lactoglobulin		α-Lactalbumin	
Adsorbent	*K*	*n*	*K*	*n*
Intact MP500	104.031	0.325	98.874	0.341
Ground MP500	254.374	0.149	131.990	0.269
MMM Chromatography	136.263	0.090	74.889	0.207

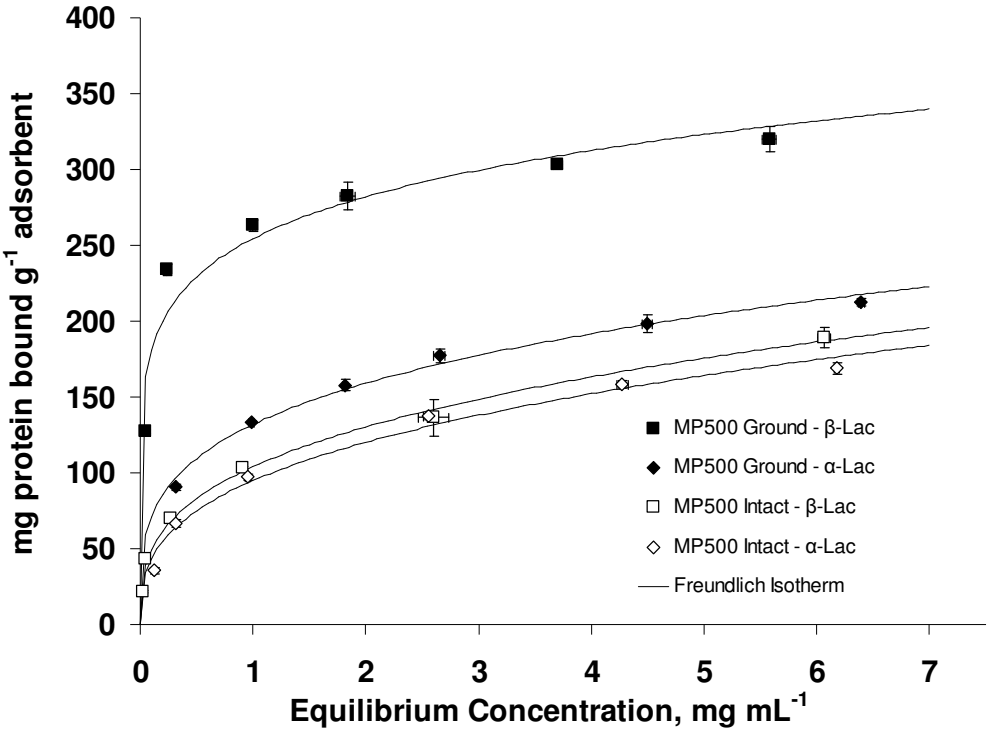


Figure 4-3: Static binding capacity of intact and ground MP500 resin for a range of β-Lac and α-Lac solution concentrations at pH 6 and room temperature (20°C). Error bars are ± one standard deviation (n=3).

As mentioned above, one reason for grinding the MP500 resin was to increase the protein adsorption capacity of the resin by reducing diffusion path lengths. Figure 4-3 shows that the equilibrium adsorption capacity for both β-Lac and α-Lac also improved dramatically after the resin was ground into particles less than 43 µm diameter. The equilibrium binding capacity of β-Lac increased from about 150 mg β-Lac g^{-1} resin to 300 mg β-Lac g^{-1} resin, and for α-Lac it increased from 140 mg α-Lac g^{-1} resin to 180 mg α-Lac g^{-1} resin. The smaller resin particles would have possessed increased total surface areas for binding and, although not reflected in equilibrium data, equilibrium would be reached faster because of reduced diffusion path lengths between the protein and adsorbent.

β-Lac, which is a larger molecule than α-Lac, showed a significant increase in binding capacity in ground resin relative to the increase in α-Lac binding. It is possible that grinding increased accessibility to internal pores, especially if there are

compartmentalized regions within the intact resin between which proteins cannot transport. The higher β-Lac adsorption than α-Lac adsorption in both intact and ground resins at pH 6.0 may be due to the charge distribution and structural rearrangements of β-Lac molecules being more favorable to the adsorption process than those of α-Lac molecules, despite their similar isoelectric points.

4.3.4 Binding capacity of membrane

Figure 4-4 shows the static binding capacities of the EVAL membrane and the MMM for β-Lac and α-Lac single protein solutions. The EVAL base membrane showed a low non-specific adsorption of β-Lac, following a linear relationship with protein concentration. Incorporation of ground MP500 resin in the base membrane increased the β-Lac adsorption capacity about sevenfold, with elution by salt. Clearly, the anion exchange resin particles in the membrane structure accounted for the increase in adsorption. The adsorption of β-Lac was higher than α-Lac for all feed solutions applied during this study. The maximum β-Lac capacity approached 150 mg β-Lac g^{-1} membrane compared to an α-Lac capacity of only 90 mg α-Lac g^{-1} membrane. The equilibrium binding capacities of these proteins at the concentrations typically found in bovine whey (3 mg mL^{-1} β-Lac and 1.2 mg mL^{-1} α-Lac (Bhattacharjee et al. 2006)) were about 140 mg β-Lac g^{-1} membrane and 75 mg α-Lac g^{-1} membrane. Goodall et al. (2008) showed a similar pattern in which the static binding capacity of β-Lac capacity is higher than α-Lac in commercial Sartobind strong and weak anion exchange membranes. However, in whey, the existence of other proteins and salts could potentially influence the adsorption process. We tested the binding of these proteins individually in solutions with the same conductivity as whey (6.14 mS cm^{-1}) and at their typical whey concentrations (figure 4-4). The binding capacities under these conditions were 80 mg β-Lac g^{-1} membrane and 20 mg α-Lac g^{-1} membrane. The binding capacity for β-Lac at the typical whey conductivity was almost as high as that of salt-free α-Lac.

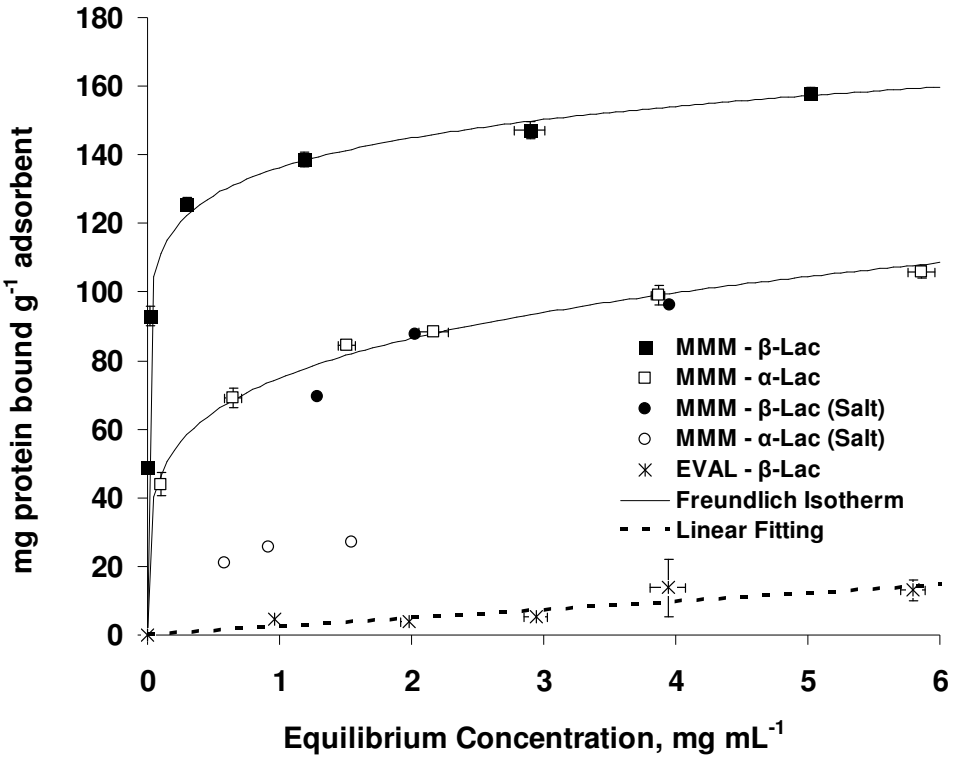


Figure 4-4: Static binding capacity of an EVAL membrane and a MMM for β-Lac and α-Lac. For β-Lac (salt) and α-Lac (salt) data, the conductivity of the binding buffer was the same as that of a typical whey solution (6.14 mS cm^{-1}). All measurements were at pH 6 and room temperature (20°C). Error bars are ± one standard deviation (n=3).

4.3.5 Batch fractionation of whey proteins using MMM chromatography

Experiments on fractionation of the major acidic whey proteins resulted in average binding capacities of 3.540 mg α-Lac g^{-1} membrane, 0.543 mg BSA g^{-1} membrane, and 75.639 mg β-Lac g^{-1} membrane (table 4-3). Recoveries of 77.7-84.6% compares well with reported values for packed bed chromatography (Doultani et al. 2003). The MMM capacity for β-Lac in whey was almost the same as for pure β-Lac binding with a similar conductivity (figure 4-4). However, the α-Lac binding capacity in whey was significantly reduced compared to its value with pure protein at the same conductivity as whey. This result is consistent with a previous study on the binding of whey proteins by chitosan, which also showed selective binding of β-Lac over

Table 4-3: Whey protein fractionation using MMM chromatography in batch adsorption at room temperature overnight.

ID	MMM mass (mg)	Protein type	Total protein in solution (mg)	Protein bound to membrane (mg)	mg protein bound g^{-1} membrane	Protein elute after elution (mg)	Recovery (%)
1	16.20	α-Lac	1.250	0.060	3.716	0.000	0.00
		BSA	0.109	0.011	0.682	0.000	0.00
		β-Lac	4.252	1.220	75.329	0.983	80.51
2	17.52	α-Lac	1.250	0.041	2.367	0.000	0.00
		BSA	0.109	0.008	0.456	0.000	0.00
		β-Lac	4.252	1.287	73.448	1.089	84.60
3	17.07	α-Lac	1.250	0.053	3.082	0.000	0.00
		BSA	0.109	0.020	1.149	0.000	0.00
		β-Lac	4.252	1.263	73.995	1.004	79.49
4	16.95	α-Lac	1.250	0.082	4.831	0.000	0.00
		BSA	0.109	0.006	0.328	0.000	0.00
		β-Lac	4.252	1.352	79.749	1.050	77.69
5	17.14	α-Lac	1.250	0.063	3.704	0.000	0.00
		BSA	0.109	0.002	0.103	0.000	0.00
		β-Lac	4.252	1.297	75.675	1.039	80.09
Average		α-Lac			3.540 ± 0.910		0
		BSA			0.543 ± 0.398		0
		β-Lac □			75.639 ± 2.474		80.48 ± 2.54

other proteins in whey at pH 6.0 (Casal et al. 2006). This behaviour may be due to the distribution of electrostatic charges on the surface of the protein. It is believed that in α-Lac molecules, most of the positively charged amino acid residues are located near each other and there is also one particularly large charge patch, consisting of a cluster of six positively charged groups (De Vries 2004). The presence of this large positively charged patch may give rise to a strong reduction in the electrostatic attraction between α-Lac and the MP500 resin, favoring competitive adsorption of β-Lac from whey.

4.3.6 Binding preference of acidic whey proteins to anionic MMM

At pH 6, all negatively whey protein like β-Lac, α-Lac and BSA has an ability to interact with anion exchanger membrane. However, in previous studies (Goodall et al. 2008), β-Lac showed most selective binding toward strong anion exchanger membrane. It even could displace other positively proteins bound onto the anionic membrane. The binding strength of three major proteins on anion exchanger membrane could be postulated according to this order: β-Lac > BSA > α-Lac (Goodall et al. 2008; Weinbrenner and Etzel 1994). To verify this order, a series of batch adsorption using whey and simulated whey was conducted. Figure 4-5 shows the amount of individual protein bound onto the anion exchange MMM from a series of initial whey solution with different dilution factor. As illustrated in figure 4-5, β-Lac is preferably bound to the anion exchange membrane and for the small area of membrane used in this experiment, it has a specific capacity of about 1 mg for β-Lac (or capacity of 21 mg β-Lac mL^{-1} MMM). We may see that when the whey was diluted 4 times (whey 4X), nearly 100% β-Lac was bound, 90% BSA bound and less than 10% α-Lac bound. In whey 8X, since β-Lac in solution was less than 1 mg, all the β-Lac was firstly bound and at this stage the remaining membrane capacity would go for another protein as shown in an increase in binding percentage (almost 80% for α-Lac and 90% for BSA).

A further experiment using a simulated whey solution with serial dilution of β-Lac concentration (i.e. α-Lac and BSA concentration kept constant) was also conducted for another verification. Figure 4-6 shows the binding capacity of the MMM for this simulated whey solution. The specific β-Lac capacity was above 1.5 mg which was

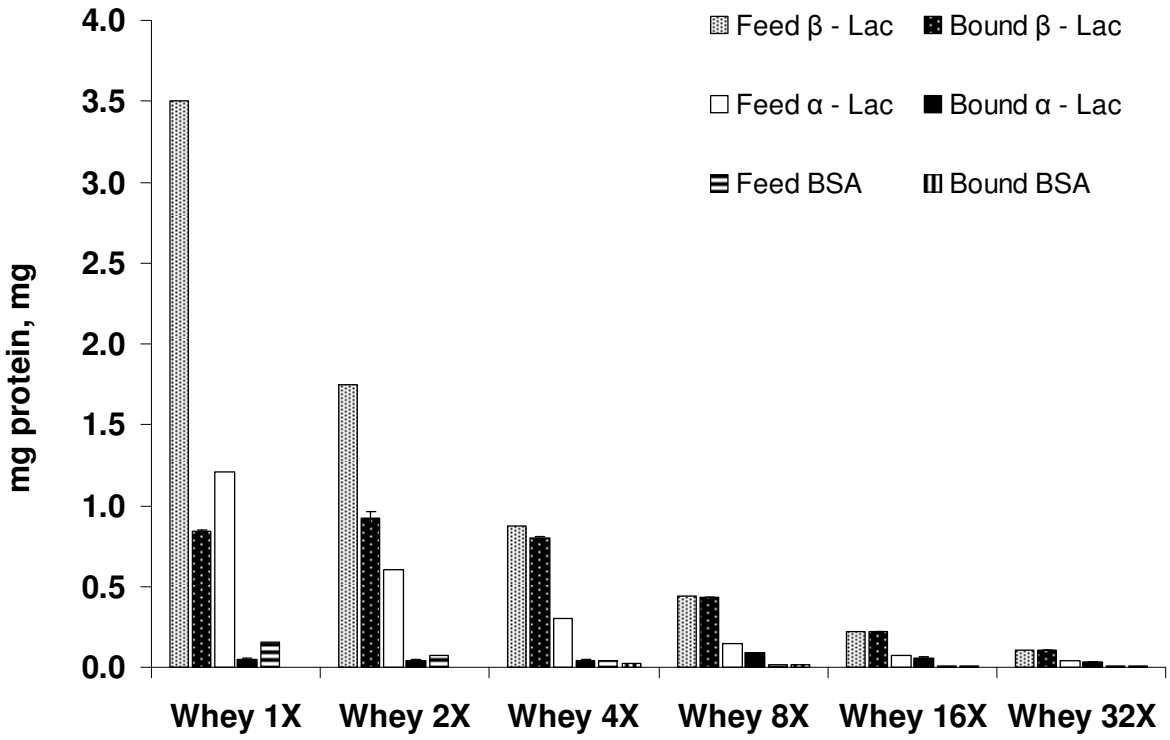


Figure 4-5: Individual protein bound onto anion exchange MMM using a 1 mL of whey solution with different initial concentration. Whey was diluted with the binding buffer in a serial dilution. Error bars are ± one standard deviation (n=3).

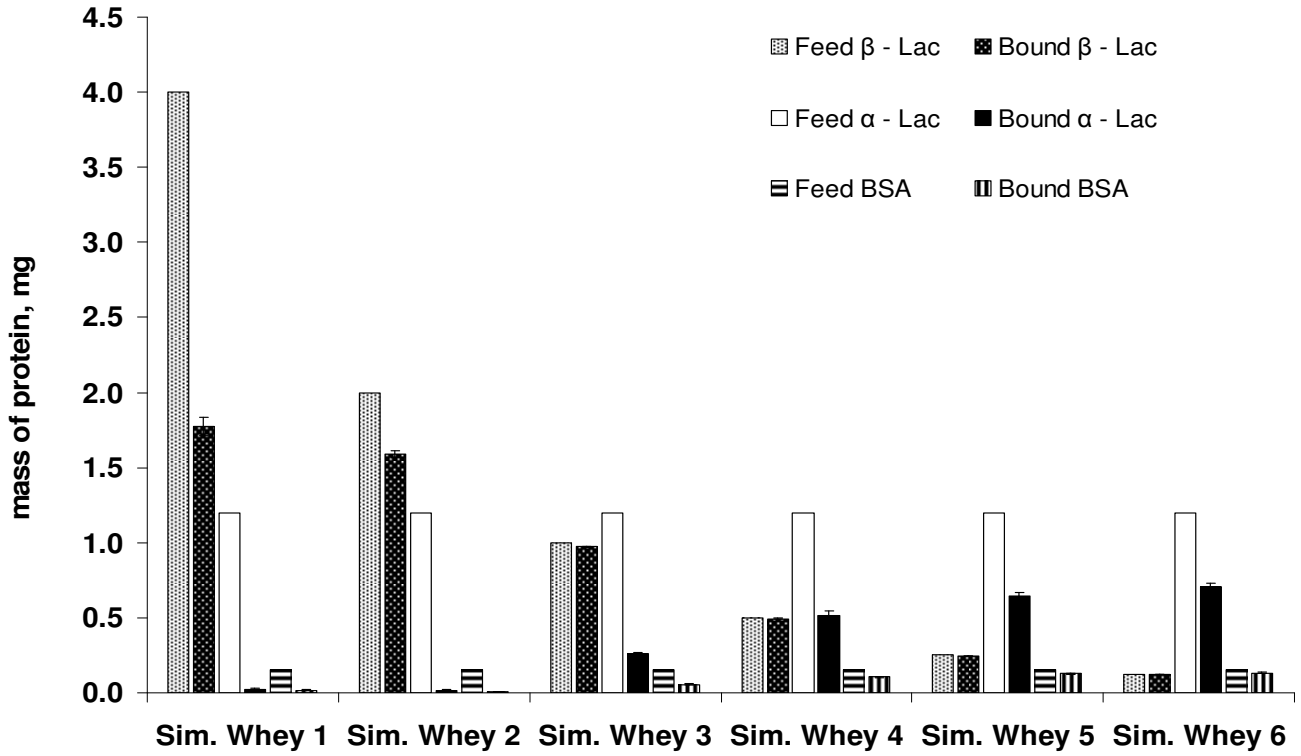


Figure 4-6: Individual protein bound onto anion exchange MMM using a 1 mL of simulated whey solution with different initial of β-Lac concentration. The concentration of α-Lac and BSA was kept constant at 1.2 mg mL^{-1} and 0.15 mg mL^{-1} respectively. Error bars are ± one standard deviation (n=3).

higher than a real whey solution previously. This could be due to the absence of ion or another minor proteins or peptide component in this simulated whey which has minimized the competitive β-Lac binding to the membrane. The pattern was similar, after one stage when there was not enough β-Lac for the MMM to bind, this specific capacity will goes to another protein preferably BSA. In the simulate whey solution 3, almost 50% of BSA bound compared to α-Lac which is only 20 % bound. The rest of solution showed almost 90% of BSA bound and the percentage of α-Lac bound was gradually increased. These results confirmed that anion exchange MMM followed a similar binding preference order of β-Lac > BSA > α-Lac as reported in the previous studies (Goodall et al. 2008; Weinbrenner and Etzel 1994).

4.3.7 Dynamic binding capacity of MMM chromatography

The capacity of the MMM for a standard protein, BSA, was 96 ± 7.4 mg BSA g^{-1} MMM or 29 ± 2.3 mg BSA mL^{-1} MMM or 0.58 ± 0.04 mg BSA cm^{-2} MMM. This compares well with the binding capacities for several adsorptive membranes reported by Roper and Lightfoot (1995) that ranged from 3 to 50 mg macromolecule mL^{-1} of membrane and with the commercial membrane Sartobind Q, which has a reported capacity of about 0.8 mg BSA cm^{-2} membrane (Goodall et al. 2008).

Figure 4-7 shows the effect of flow rate on the β-Lac dynamic binding capacity of the MMM. A flow rate of 5 mL min^{-1} in the MMM corresponds to a permeate flux rate of just under 200 LMH, which is a high flux rate for a membrane. The results show that flow rate does not have a significant effect on dynamic binding capacity, which is most likely due to the high rate of convective transport of the protein within the membrane and minimal diffusive path lengths within the membrane and the finely ground resin particles. This is a characteristic of almost every adsorptive membrane system reported in the literature and is one of the main advantages of this technology, an advantage apparently not reduced by the incorporation of resin particles rather than chemical modification of the base membrane.

It is interesting to note that the dynamic binding capacity of β-Lac in whey is higher than that of pure β-Lac prepared in the binding buffer as shown in figure 4-7 (protein concentration of 3 mg mL^{-1}).

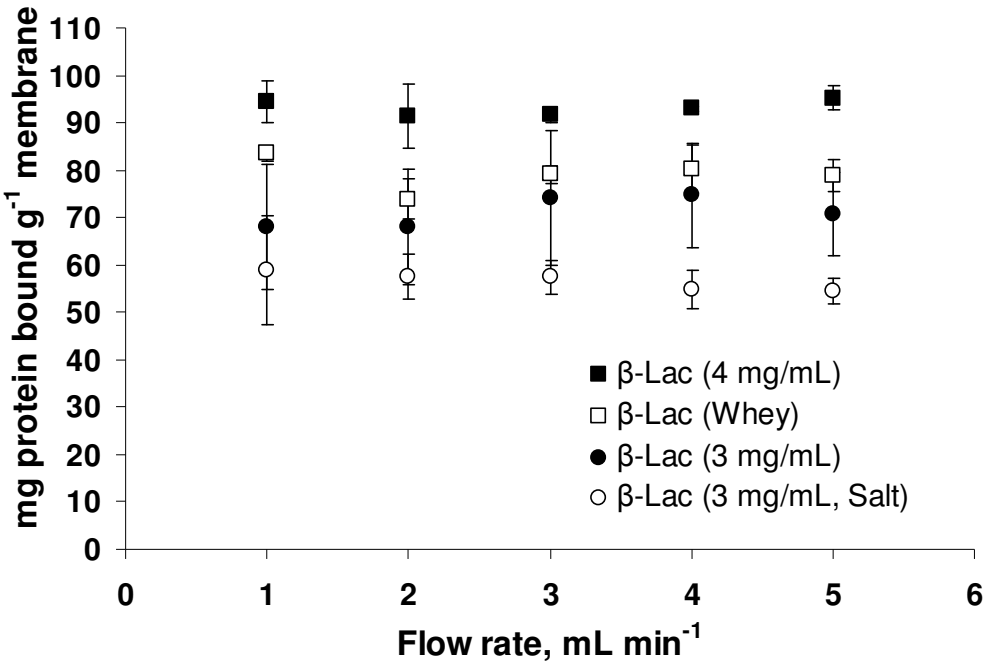


Figure 4-7: Effect of flow rate on the β-Lac dynamic binding capacity of MMM chromatography for pure (salt-free) protein solution of 3 mg mL^{-1} and 4 mg mL^{-1}, whey and a pure protein solution 3 mg mL^{-1} having the same conductivity as whey (6.14 mS cm^{-1}). All measurements were at pH 6 and room temperature (20°C). Error bars are ± one standard deviation (n=3).

We hypothesized that this difference might be due to the salt concentration in whey allowing a greater surface mobility of the bound protein near pore entrances, thus lowering steric hindrance of subsequent binding events. Therefore we adjusted the conductivity of the pure β-Lac solution to the same value as found in whey and tested its dynamic binding capacity (labelled 'β-Lac (3mg mL^{-1}, salt)' in figure 4-7). However, the dynamic binding capacity for pure β-Lac (salt) was much lower than that of pure β-Lac prepared in either low-salt binding buffer (3 mg mL^{-1}) or actual whey.

Due to the variation of the β-Lac concentration in whey, there is a possibility that its concentration may have been higher than 3 mg mL^{-1}. Since higher initial protein concentration will contribute to higher binding capacity, especially in the region below its saturation point, the dynamic binding capacity of the MMM was checked

using a higher initial β-Lac concentration. As expected, the dynamic binding capacity at 4 mg mL^{-1} was higher than at 3 mg mL^{-1} and in whey (figure 4-7), consistent with the concentration in whey being between 3 and 4 mg mL^{-1} (table 4-1). The same membrane was used after cleaning between runs for all dynamic capacity experiments and figure 4-7 shows that there was no significant loss of capacity with re-use of the membrane.

When considering how these results relate to industrial scale applications, a suitable size basis must be chosen for calculating productivity (i.e., production rate of protein per unit size of equipment). Productivity comparisons between membrane chromatography and packed-bed chromatography on the basis of unit volume is not straightforward, as membrane volume alone does not account for hold-up volume in the membrane housing, which is typically about four times the membrane volume. Resin volume figures, on the other hand, usually include void volume (approximately 40%) so approximate the total column volume. For membrane separations, equipment is usually sized by membrane area so productivity calculations on this basis are probably the most appropriate, unless comparisons with packed-bed chromatography are desired.

In the following productivity estimates, figures calculated using all three bases are given. A 1,000 m^2 MMM has a volume of about 200 L (at 200 µm thickness) and from our results this has an adsorption capacity of about 5 kg of protein, equivalent to the β-Lac content of 1,666 L of whey. If we allow for a typical chromatographic cycle of 5 CV equilibration buffer, 1,666 L feed loading, 2 CV wash, 1 CV elution and 2 CV regeneration, this amounts to 3,366 L of permeate per cycle. At a modest 50 L m^{-2} h^{-1} permeate flux rate, this would have a total cycle time of approximately 4 min. Productivity on the basis of membrane volume is therefore 371 g $L^{-1}_{membrane}$ h^{-1}. A 1,000 m^2 spiral-wound membrane module would typically have a hold-up volume of 1 m^3 (Cheryan 1998) so this productivity corresponds to 74 g L^{-1}_{module} h^{-1} on a membrane module volume basis or 74 g m^{-2} h^{-1}on a membrane area basis. These values compare well with reported values in the range 18-60 g L^{-1}_{resin} h^{-1} for laboratory-scale batch and packed-bed chromatography systems for whey protein isolate production and 240 g L^{-1}_{resin} h^{-1} estimated for industrial-scale systems (Doultani et al. 2003).

4.4 Conclusions

MMMs, which incorporate adsorptive particles during membrane casting, can be prepared simply and have performances that are competitive with other membrane chromatography materials. MMM prepared in this study showed excellent potential for a whey protein fractionation application, particularly for selective binding of β-Lac. The membrane had a defect-free structure and provided a high binding capacity for β-Lac in whey solution, compared with other proteins. The MMM had maximum equilibrium binding capacities of 150 mg β-Lac g^{-1} membrane (36 mg mL^{-1} membrane) and 90 mg α-Lac g^{-1} membrane (27 mg mL^{-1} membrane) in individual pure protein experiments. In batch fractionation of whey, the MMM had almost the same binding capacity for β-Lac as it did for pure β-Lac. The dynamic binding capacity of β-Lac in whey solution was not affected significantly by flow rate and had a value of about 80 mg β-Lac g^{-1} membrane (24 mg mL^{-1} membrane), which is promising for the selective capture of β-Lac from bovine whey. This is the first reported application of MMM chromatography to a dairy feed stream.

5 Recovery of lactoferrin from whey using cross-flow cation exchange mixed matrix membrane chromatography

5.1 Introduction

In this chapter, a cation exchange MMM was developed by incorporating SP Sepharose Fast Flow resin (GE Healthcare Technologies) into an EVAL membrane. The static binding capacity of SP Sepharose based adsorbent in different formats: intact resin, ground resin and membrane were measured for pure LZY and LF. This cationic MMM is expected to bind LF from whey solution. The feasibility of cross-flow MMM chromatography for LF recovery was demonstrated and the performance of the MMM was evaluated, in terms of protein binding capacity, permeate flux rate, LF recovery and purity. CLSM was used to identify the location of protein adsorption within the cationic MMM.

5.2 Materials and methods

5.2.1 Chemicals

All chemicals used for making a membrane, buffer preparation and proteins used to test the membrane performance were described in detail in section 3.1.

5.2.2 Cationic resin

Three types of Amberlite® cation exchange resin, Amberlite IRC50, Amberlite IRP64 and Amberlite CG120 (Sigma), and two types of Lewatit® cation exchange resin, Lewatit SP112 and Lewatit CNP80 (Lanxess), were selected for screening as potential adsorbents for incorporation into the mixed matrix membrane. Low cost and convenient availability were the main criteria for selection.

5.2.3 Preparation of mixed matrix membranes

Mixed matrix membranes were prepared following procedures reported previously in section 3.2 but using SP Sepharose resin as the embedded adsorbent. A homogenous EVAL polymer solution, consisting of 15 wt% EVAL polymer and 15 wt% 1-octanol in DMSO was prepared by continuous stirring at about 60°C for several hours until all EVAL pellets were completely dissolved. SP Sepharose resin was ground using an ultra centrifugal mill (Retsch ZM100, Haan, Germany) and screened to obtain a particle fraction that passed through a 38 µm stainless steel mesh. Ground resin was added to the prepared polymer solution to make a 30% weight fraction (relative to the EVAL content in the polymer solution) and this mixture was stirred until a homogeneous casting slurry was obtained. A flat sheet MMM was cast on a glass plate from the slurry, as previously described (section 3.2) and the wet MMM was then freeze-dried to remove water without affecting the structure of the membrane.

5.2.4 Preparation of whey

Whey was prepared from skim (0.05% fat) milk purchased from the local retail store as previously described in section 3.3.

5.2.5 Static binding experiment

The static binding capacities of adsorbents in different forms (i.e. intact, ground and membrane forms) were measured using single protein solutions. Because LF is expensive, most capacities were determined using LZY. 20 mM sodium phosphate buffer, pH 6.0, was used as a binding buffer and 1 M NaCl was added to this for elution buffers. The amount of adsorbent used in static experiments depended on the resin form and was expressed on a dry mass basis. During cation resin scouting experiments, 20 mg of intact resin was used. Pre-equilibrated resin was incubated in small Eppendorf tubes with 1 mL of protein solution at an initial concentration of 1 mg mL^{-1}. Resin and liquid in these tubes were gently mixed by inversion throughout binding for at least 12 h at room temperature. The tubes were then spun in a centrifuge at 16,100 *g* for 5 min and the supernatant was discarded and replaced with the elution buffer. Elution was carried out by inversion mixing for another 12 h at room temperature. Protein concentrations in supernatants were assayed by

spectrophotometer (as described below). The binding capacity of each adsorbent was determined by the difference between initial and the final total solution protein content per mass of adsorbent. All experiments were carried out at least in triplicate.

To determine the equilibrium static binding capacity, 2 mg of intact and ground SP Sepharose resin were used. For membrane measurements, pieces of MMM with dimensions 12 mm × 22 mm were used, corresponding to a mass between 17 and 20 mg. These pieces of MMM had a volume of 5.3×10^{-2} mL. The adsorbent was incubated with 1 mL of known concentrations of protein solution in a series from 1 to 8 mg mL^{-1}. The equilibrium concentration was measured after 12 h and the maximum adsorbent capacity was calculated by fitting data to the Langmuir isotherm (equation 3-2 in previous section 3.5).

To determine protein recovery after adsorption, the MMM was incubated with 1 mL of 1 mg mL^{-1} of either LF or LZY solution for 12 h, then surface liquid was removed from the membrane by patting with a tissue and the MMM was transferred to a new tube containing elution buffer for another 12 h. The concentration of protein in both solutions was assayed and the recovery was calculated as the percentage of protein in the elution fraction per protein bound on the membrane. Various elution buffer NaCl concentrations from 0.25-1.0 M were tested for LF recovery from the membrane.

5.2.6 Single protein assay

Concentrations of LF and LZY were determined by measuring absorbance at 280 nm using a UV/Visible spectrophotometer (Model Ultraspec 2100 Pro, Amersham Biosciences, Amersham, England) as in section 3.7.1. A standard curve was developed from six standard concentrations from 0 to 1 mg mL^{-1}. Unknown samples were diluted with reference buffer to within the absorbance range of the standard curve.

5.2.7 Lactoferrin assay in whey fractions

The LF concentrations in whey and cross flow fractions were assayed using a RPC column following the method established by Palmano and Elgar (2002), using a 1

mL Resource™ RPC column (GE Healthcare Technologies) at a flow rate of 2 mL min^{-1}. The assay was run on an AKTAexplorer™ 10 liquid chromatography system controlled by Unicorn™ 4.0 software (GE Healthcare Technologies) with 500 µL samples manually injected through a sample injection loop. Details of the protocol were given in section 3.8.

5.2.8 Membrane porosity and gel electrophoresis

The membrane porosity was measured according to previously published methods (Avramescu et al. 2003a; Saiful et al. 2006), described in detail at section 3.4. For qualitative analysis, some of the protein fractions were analyzed by SDS-PAGE under reducing conditions, similar to the protocol described in section 3.9 except the gel used was 4-12% Bis-Tris Criterion™ XT precast gel (Bio-Rad Laboratories, Hercules, CA).

5.2.9 Protein labeling

Protein for CLSM experiment was labeled with fluorescein isothiocynate (FITC) dye from Sigma (St. Louis) according to the protocol recommended by the supplier. LZY was dissolved in 100 mM sodium carbonate buffer pH 9.0 at 2 mg mL^{-1} and FITC was dissolved in anhydrous DMSO at 1 mg mL^{-1}. Dye was added at a ratio of 50 µL/mL of protein solution. The reaction was allowed to proceed under continuous stirring for at least 8 h at 4 °C and terminated by adding ammonium chloride to a final concentration of 50 mM, under stirring for another 2 h. The protein-dye conjugate was separated from non-reacted dye on a gel filtration column, simultaneously exchanging into 20 mM sodium phosphate, pH 6.0 buffer. The molar dye to protein ratio (D/P) was determined by measuring the absorbance of the protein-dye conjugate at wavelengths of 495 nm and 280 nm. The absorbance at 495 nm corresponding to the FITC dye was divided by the absorbance value at 280 nm, which corresponds to the protein absorbance, to give the D/P ratio value. The D/P value for labeled LZY was 0.16.

5.2.10 Confocal laser scanning microscopy

A small piece of equilibrated membrane (paper hole punch size) was incubated overnight at room temperature with 1 mL of FITC-LZY solution prepared as above. The membrane was washed several times with binding buffer before placing it on a clean, dry glass slab. A drop of glycerol was dropped onto the membrane and another small glass slab was used to cover the membrane. CLSM images were acquired using a Leica TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany) with a 63X objective lens. An argon laser provided excitation of FITC at 488 nm. The image resolution was 1024 × 1024 pixels and up to 28 images were taken at depths separated by 1 µm throughout the membrane thickness.

5.2.11 Cross-flow system

Cross-flow experiments were run using an AKTAcrossflow™ (GE Healthcare Life Sciences) tangential flow filtration system under the control Unicorn 5.11 software. The membrane setup was described in section 3.10 previously.

Cross-flow experiments were conducted in triplicate, using one, two and three pieces of membrane. During loading, 150 mL of whey was circulated past the membrane and both retentate and permeate streams were recycled back into the feed reservoir. Whey loading was completed when the cumulative permeate volume reached 300 mL. Throughout the cross-flow experiments, the feed flow rate was kept constant at 50 mL min^{-1} and the permeate flux was operated at 100 L m^{-2} h^{-1} (LMH), except during the whey loading, when the permeate flux was reduced to 50 LMH. After loading, the retentate side of the system was drained and flushed several times with about 1000 mL of binding buffer. 200 mL of fresh binding buffer was then transferred to the reservoir for the membrane washing step and circulated past the membrane under constant flux conditions as above until the cumulative permeate volume reached 150 mL. The remaining fluid in the reservoir was then drained, filled with 120 mL of elution buffer and circulated past the membrane under constant flux conditions as above until the permeate volume reached 90 mL. Retentate samples and permeate fractions were collected for analysis at each step. LF recovery (the percentage of mass of LF in elution per mass of LF in feed whey) was calculated and the TMP profile during whey loading was also recorded. Fresh membranes were used

for each cross-flow experiment. Before starting an experiment, each membrane was pre-treated with 0.5 M NaOH for 30 min and flushed with water until a neutral pH was obtained.

5.3 Results and discussion

5.3.1 Cation resin scouting

Figure 5-1 shows the binding capacity of LZY on the cation resins and the percentage of bound LZY recovered during salt elution. The amount of LZY bound on Amberlite resins was relatively low compared with that on Lewatit resins and most resins yielded low protein recoveries, indicating the presence of irreversible protein adsorption. Although Amberlite CG120 had high recovery (90%), the binding capacity was less than 5 mg LZY g^{-1} resin. In our hands, neither Amberlite nor Lewatit cation exchange resins performed well, contrary to recovery values of over 90% reported in previous work for Lewatit CNP80 (Saiful et al. 2006) and Lewatit SP112 (Avramescu et al. 2003a) resins. Therefore, in this study we decided to use SP Sepharose Fast Flow (GE Healthcare Technologies), which was designed for high resolution protein purification.

5.3.2 Confocal imaging

Interest in the use of CLSM in membrane research originated from its novel application to the characterization of resin bead chromatography media. Hubbuch and Kula (2008) recently reviewed the use of CLSM as an analytical tool in chromatography research. In the membrane field, CLSM has been used for various purposes, such as to visualize protein binding within the chromatographic membrane structure (Reichert et al. 2002; Wang et al. 2008) and to characterize the morphology of microfiltration membranes (Charcosset and Bernengo 2000; Charcosset et al. 2000) as well as to characterize fouling mechanisms in membranes (Ferrando et al. 2005).

To date, mainly commercial membrane chromatography materials have been examined using the CLSM technique (Reichert et al. 2002; Wang et al. 2008;

Wickramasinghe et al. 2006). For mixed matrix membranes, it is of interest to see whether or not the resin particles embedded within the membrane scaffold can perform their function. Therefore, the objective of the CLSM experiment was to visualize LZY binding on the SP Sepharose particles embedded inside the MMM structure at different depths throughout the membrane thickness. Figure 5-2 shows a CLSM image of a selected area of the MMM at different membrane depths (z-axis) from the top towards the bottom of the membrane. Clearly, LZY binding occurs only on the SP Sepharose resin particles within the MMM and no nonspecific binding of LZY to the base EVAL membrane was observed. Furthermore, a greater number of resin particles were found below the surface than at the top of the membrane and, as expected, particle fracture during grinding did not inhibit binding. The membrane thickness was about 200 µm but an attempt to scan deeper than 17 µm resulted in a loss of fluorescent signal.

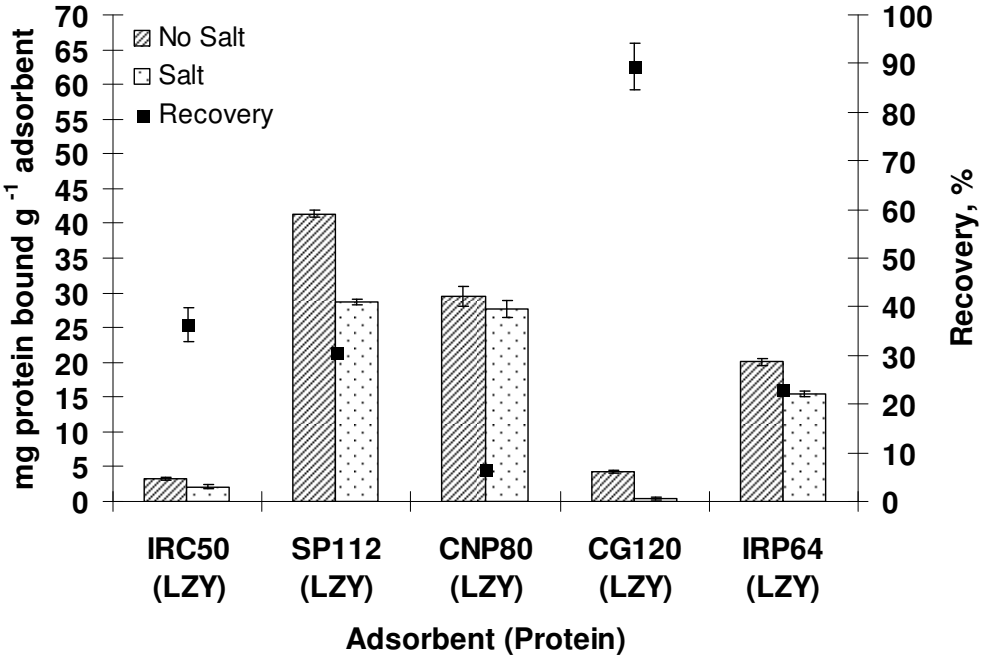


Figure 5-1: Adsorption and desorption of lysozyme on Amberlite and Lewatit cation exchange resins. The binding buffer was 20 mM sodium phosphate, pH 6.0 (no salt) and the elution buffer was 1 M NaCl in binding buffer. A triplicate sample was used for each resin. Error bars are ± one standard deviation (n = 3).

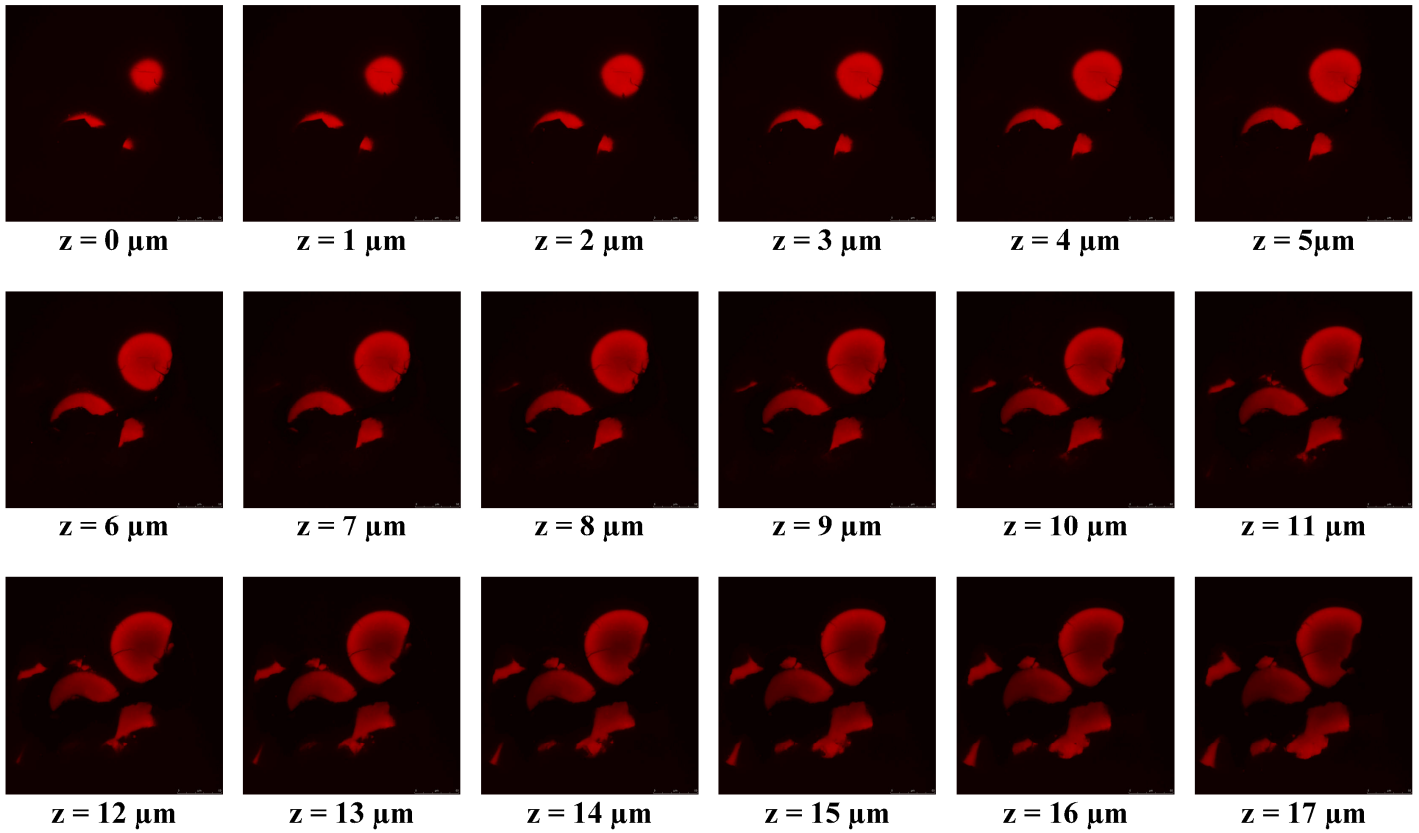


Figure 5-2: CLSM images of a cation exchange MMM at increasing depths through the membrane. LZY was labeled with FITC dye, showing binding to the SP Sepharose resin particles within the membrane matrix.

5.3.3 Isotherms for protein adsorption

Figure 5-3 shows the protein adsorption isotherms for intact and ground SP Sepharose resin and a MMM developed using the resin. A Langmuir adsorption isotherm was fitted to the equilibrium protein capacity data of the adsorbents in figure 5-3, using a least-squares regression method. The maximum LZY binding capacities calculated using the Langmuir isotherm were 832 mg g^{-1} intact resin, 1032 mg g^{-1} ground resin or 262 mg g^{-1} membrane (dry resin and membrane weight bases).

In Chapter 4 work on anionic MMMs, the β-Lac binding capacity was doubled when the intact resin was ground into a smaller size. This increment was probably due to the increased total surface area for binding and the improvement of protein accessibility to the internal pores within the resin. The current result, however, demonstrates that grinding the SP Sepharose resin resulted in a 24% improvement in LZY static binding capacity. LZY apparently has high accessibility to the internal pores of the intact SP Sepharose structure but a small fraction of the intact ion exchanger, not normally accessible to the protein, was made available upon grinding, contributing to an increase in protein binding capacity. The main objective of grinding the SP Sepharose resin was to improve the adhesion and homogeneous distribution of the resin within the membrane matrix rather than to change the adsorption capacity of the particles. However, smaller resin particles may also improve the dynamic binding capacity in MMM because the diffusion path lengths to the internal ion exchange sites would decrease and there would be a simultaneous increase in the interfacial surface area between permeate and resin as demonstrated in Chapter 4 previously.

Thirty weight percent of SP Sepharose ground resin (relative to the mass of EVAL polymer) was added to the casting polymer solution to prepare the cationic MMM. This loading percentage is quite low compared with the loading percentages (50 wt% of Lewatit MP500 anion resin in Chapter 4 or 65 wt% Lewatit CNP80 cation resin (Saiful et al. 2006)) used in other MMM preparations. SP Sepharose resin swelled significantly more in the solvent than the Lewatit resin so at higher resin loadings, the casting polymer solution became highly viscous and was difficult to cast as a

membrane. Based on figure 5-3, the cationic MMM had a maximum static binding capacity for pure LF of 384 mg g^{-1} membrane, 155 mg mL^{-1} membrane or 3.11 mg cm^{-2} membrane. LZY gave values of 262 mg g^{-1} membrane, 79 mg mL^{-1} membrane or 1.58 mg cm^{-2} membrane. These capacities are competitive with the values for other membranes reported in the literature. Non-specific protein binding to the EVAL base membrane was relatively low and can be neglected (Chapter 4 and Saiful et al. 2006). The LZY binding capacity achieved was similar to the values reported for cation exchange hollow fibers (84 mg mL^{-1} membrane) by Camperi et al. (1999) and was higher than for Whatman (46.5 mg mL^{-1} membrane, Lin and Suen 2002) and Sartobind S (15.6 mg mL^{-1} membrane, Fang et al. 2004) commercial cation exchange membranes. Although Saiful et al's (2006) cation MMM showed the highest capacity of 147 mg mL^{-1} membrane, they used a 65 wt% cationic resin loading, more than double the SP Sepharose resin loading used in the current study.

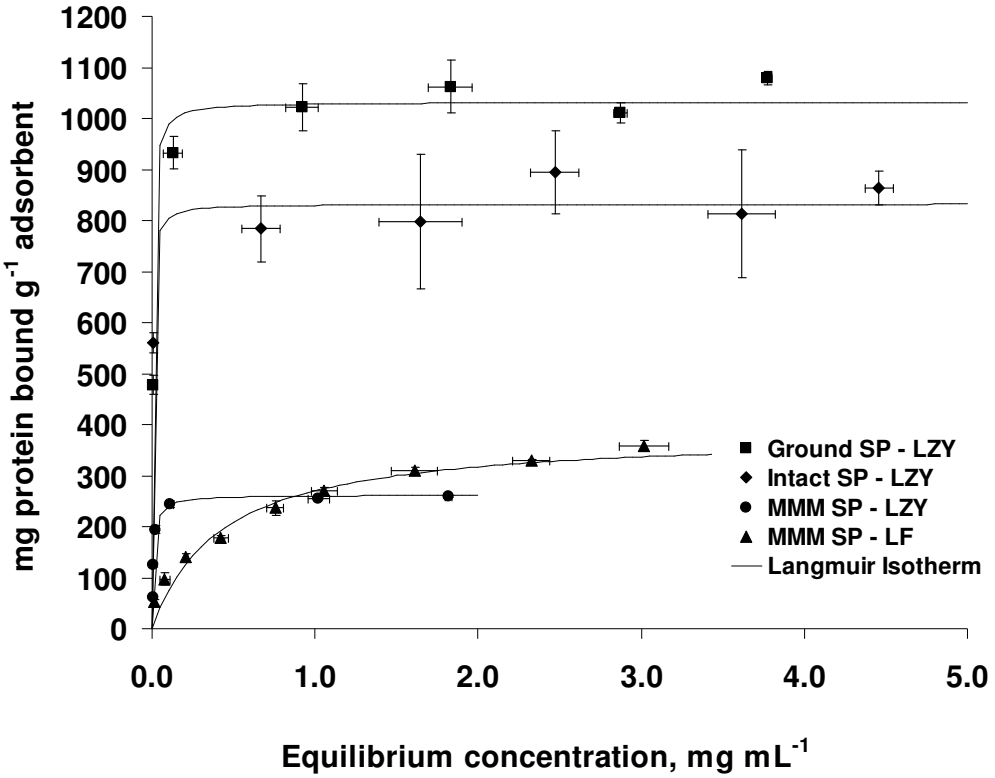


Figure 5-3: Static (equilibrium) binding capacities of intact and ground SP Sepharose resin and a cation exchange MMM for LZY and LF. The adsorption data was fitted using the Langmuir isotherm using triplicate adsorbents samples. Error bars are ± one standard deviation (n = 3).

The maximum binding capacity of the MMM for LF was 155 mg mL^{-1}, which is higher than the value of 111 mg mL^{-1} reported for a dye affinity hollow fiber membrane (Wolman et al. 2007). At an LF equilibrium concentration of 0.12 mg mL^{-1} (within the normal LF concentration range found in whey), the cationic MMM showed a capacity of 85 mg g^{-1} membrane or 30 mg mL^{-1} membrane.

5.3.4 Elution experiments

NaCl concentrations from 0.25 to 1 M were tested for their ability to desorb bound LF from the MMM. The results shown in figure 5-4 demonstrate that at least 0.75 M salt was required to achieve more than 80% LF recovery desorption from the membrane after protein binding and that close to 100% recovery of LZY was achieved at 1.0 M NaCl. Therefore, in subsequent cross-flow experiments, 1M NaCl was adopted to ensure maximum recovery.

5.3.5 Cross-flow filtration of whey on membrane chromatography

According to Lin and Suen (2002), a plate-and-frame module with a flat sheet membrane is the best design for adsorptive membranes for large scale separations. Advantages include high capacity, convenience of scale up and the capability of combining filtration and adsorption in one step (Lin and Suen 2002; Tsai and Suen 2001).

Figure 5-5 shows an example chromatogram from a cross-flow experiment. Based on permeate volumes (excluding the time taken to fill the reservoir and to flush the system), the equilibration step took 36 min, the whey loading step took 72 min, the washing step took 18 min and the final elution step took 11 min. The total time was therefore about 137 min for a total permeate volume of 840 mL. In figure 5-5, it is clear from the UV and conductivity curves that less time is required for the equilibration, wash and elution steps, potentially saving approximately 370 mL of permeate or 47 minutes. In that case, the process would take approximately 90 minutes, 80% of which would be for loading.

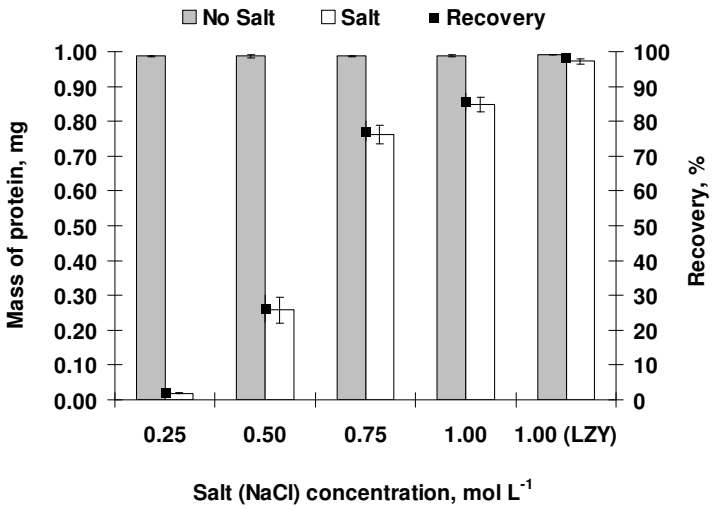


Figure 5-4: Adsorption and desorption of LF at various salt concentrations and LZY at 1 M NaCl for a cation exchange MMM. 1 mg of protein (1 mL of 1 mg mL^{-1} protein solution in 20 mM sodium phosphate pH, 6.0 (no salt)) was adsorbed to the membrane and recovered using different salt concentrations during elution. A triplicate sample was used for each salt concentration. Error bars are ± one standard deviation (n = 3).

The pure water flux for the cationic MMM is shown in table 5-1. Water flux values for flat sheet membrane chromatography materials are rarely reported in the literature. For a general comparison, the water flux value for the single membrane layer was normalized with pressure to give a value of 16.72 LMH kPa^{-1}. The cationic MMM prepared by Saiful et al. (2006) had a water flux value of 10 LMH kPa^{-1}, measured in dead end filtration mode. A polyvinyl difluoride (PVDF) microfiltration membrane with pore size 0.5 µm had a water flux value 5.22 LMH kPa^{-1} (Brisson et al. 2007). From the water flux value obtained, we believe that the cation exchange MMM prepared in this study has an adequate pore size for permeation of whey proteins. In addition, the cation resin particles embedded within the membrane apparently did not cause significant resistance to fluid flow through the membrane. The cationic MMM had a porosity value of about 72%, which is quite similar to other types of MMM reported in previous studies (Avramescu et al. 2003b; Saiful et al. 2006).

Figure 5-6 shows the TMP profile of the cross-flow system as a function of accumulated permeate volume during the whey loading step. A constant flux value of 50 LMH was used during the loading step to ensure that system backpressure was maintained below the maximum allowable value of 520 kPa. The whey circulation step took about 72 min to complete. Higher flux values could possibly have been used to reduce the process time, with a corresponding increase in the TMP but there is danger that a critical flux rate might be exceeded and cause severe fouling (Bacchin et al. 2006; Field et al. 1995). An alternative method to reduce the circulation time would be to operate at a constant TMP just below the maximum allowable system pressure. An attempt to use a TMP of 200 kPa, however, gave a significant flux loss (data not shown), supporting the existence of a critical flux rate beyond which significant fouling occurs. With three membrane layers in the module, the flux rate decline occurred immediately at 200 kPa TMP and eventually leveled off to a value of around 10 LMH, which is far below the flux rates acceptable for industrial membrane processing. Therefore, a moderate, constant flux of 50 LMH was selected for subsequent studies and the TMP profiles and binding properties of a varying number of membrane layers were studied in a plate-and-frame module.

Table 5-1: Average[1] water flux, LF binding capacity and recovery for cation exchange mixed matrix membranes using differing numbers of membranes in a plate-and-frame module. The total amount of LF in 150 mL of whey feed was 17.63 mg.

Number of membranes in module	Water flux at a transmembrane pressure of 25 kPa, L m^{-2} h^{-1}	Mass of LF in elution fraction, mg	Recovery,[2] %
1	418.0±19.4	11.51±1.25	65.31±7.06
2	255.6±17.5	13.75±0.11	78.02±0.62
3	188.0±31.2	15.46±0.62	87.71±3.53

[1] Average values ± one standard deviation (n = 3)

[2] Recovery = (mass of protein in elution fraction/mass of protein in feed) x 100%

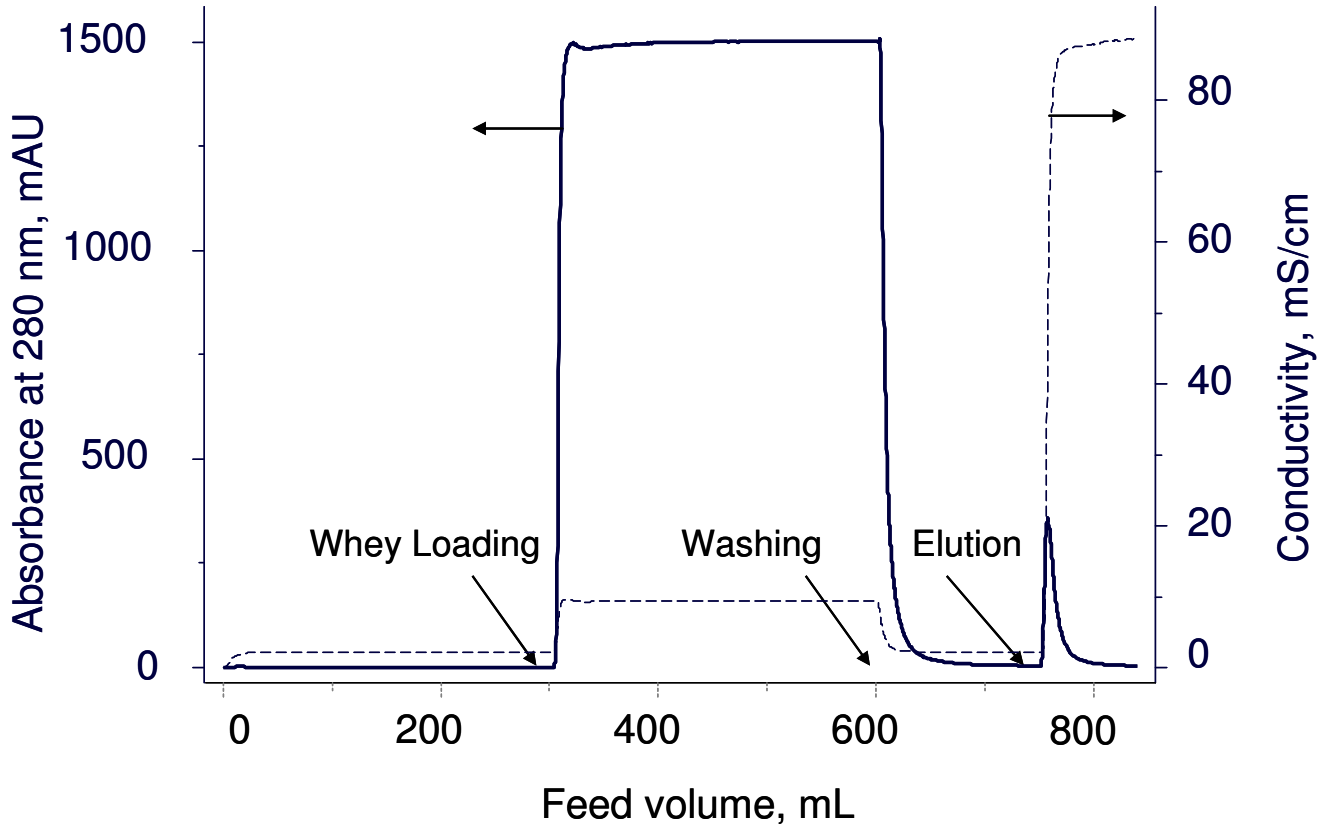


Figure 5-5: Chromatogram of a typical cross flow experiment (three pieces membrane) recorded by the AKTAcrossflow™ system. The solid line represents the absorbance value and the dotted line represents the conductivity in the permeate.

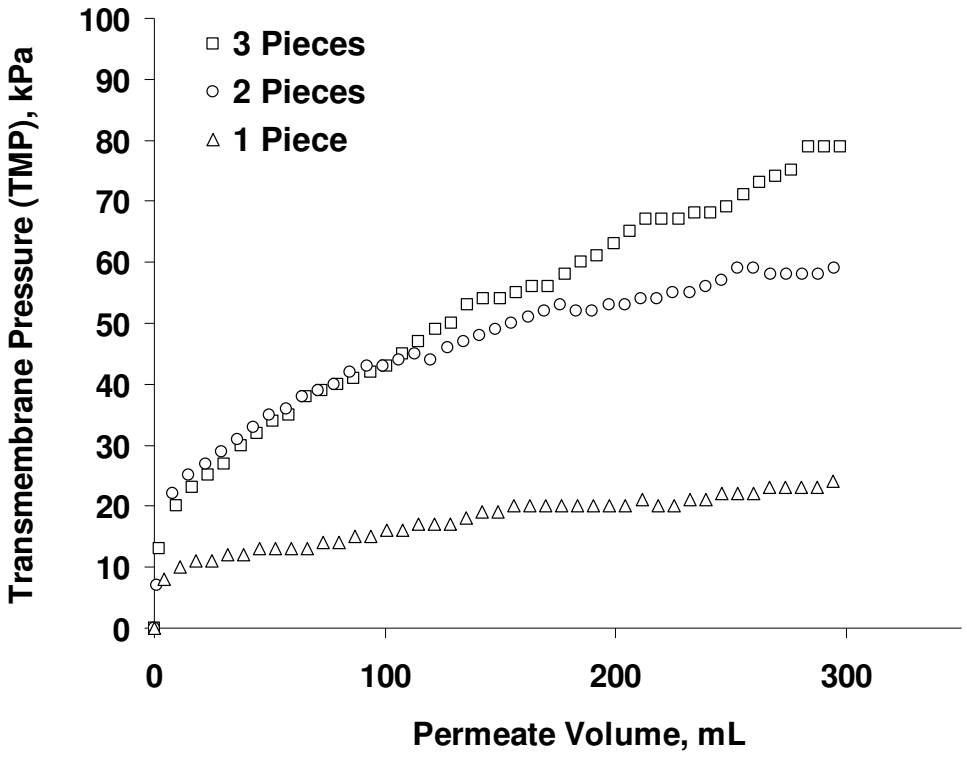


Figure 5-6: Typical TMP profiles during whey loading for differing numbers of cation exchange MMM in the module. 150 mL of whey was passed through the membrane, with both permeate and retentate recycled to the feed solution.

As shown in figure 5-6, there was no significant increase in TMP for one piece of membrane up to an accumulated permeate volume of 300 mL. The use of two or three pieces of membrane caused a gradual increase in TMP under constant flux control, although the values remained below the maximum system backpressure. The increase in TMP during permeate accumulation is likely to have been caused by gradual membrane fouling. Advances in fouling control techniques such as the use of turbulence promoters, pulsed/reversed flow, additional electrical fields or addition of surfactants to the feed solution (Chilukuri et al. 2001; Wakeman and Williams 2002) may be applicable in the development of cross-flow membrane chromatography but consideration of fouling is beyond the scope of this chapter.

Although unusual in conventional packed-bed chromatography columns and dead-end adsorptive membrane processes, recycle of the permeate stream to the feed tank

can easily be allowed for in cross-flow operation for multiple passes through the adsorbent to maximize adsorption. Increasing the number of membrane layers contained in one module was expected to increase the protein binding capacity of the membrane chromatography system. In addition, using multiple layers of membrane should provide a more uniform flow distribution, with any defects in one layer compensated for by the flow through subsequent layers (Suen and Etzel 1992).

Table 5-1 shows the amount of LF bound on the cationic MMM and recovered from 150 mL of whey for 1, 2 and 3 membrane layers. Recovery was defined as the percentage of the LF originally contained in the whey feed obtained in the eluted fraction. The concentration of LF in the feed whey was 117.50 ± 14.55 mg L^{-1}. Three pieces of membrane gave an average recovery of 88%, which is quite competitive with previous studies in other systems. For example, Lu et al. (2007) reported 82% recovery using ultrafiltration, followed by ion exchange chromatography to purify LF from bovine colostrum. Other studies reported LF recoveries of about 88% from 1000 L of whey using a 2 m^2 commercial Sartobind membrane module (Plate et al. 2006) and 89% using dye affinity membrane chromatography from 25 mL of whey (Wolman et al. 2007). The LF recovery could be increased further by adding more membrane layers, with diminishing economic returns eventually giving an optimum number of membrane layers and binding capacity (Lin and Suen 2002). However, optimization of economics is beyond the scope of the current study.

Figure 5-7 shows an SDS gel image of LF in the fractions collected during the cross-flow chromatography run. A single band of LF can be seen in the elution fraction from each of the cross-flow experiments. The RPC chromatogram for the cross-flow fractions sampled during the single membrane experiment is shown in figure 5-8, with LF eluting at approximately 14 mL. The results show that the acidic proteins β-Lac, α-Lac and BSA are removed in the wash steps and that the elution fraction contains mainly LF.

5.4 Conclusions

A cation exchange MMM, designed for the recovery of LF from acid whey, was prepared using ground SP Sepharose resin cast into an EVAL base membrane. The

static LF binding capacity of the MMM had a maximum protein capacity of 384 mg g^{-1} membrane, 155 mg mL^{-1} membrane or 3.11 mg cm^{-2} membrane, which is competitive with commercially available membranes and other cationic adsorptive membranes reported in the literature.

The chromatographic steps were carried out using a cross-flow operation to minimize concentration polarization and enhance the binding capacity of the MMM by recycling both permeate and retentate streams into the feed during the whey loading step. Several layers of MMM were inserted into a plate-and-frame module, each with an effective membrane area of 50 cm^2. Three pieces of MMM in the module gave an average LF recovery of 88% from 150 mL of feed whey. The eluted LF had a high purity, as shown by SDS-PAGE and RPC. This is the first reported study of cross-flow cationic MMM chromatography for recovery of LF from whey. The results indicate that mixed matrix membranes, which are easily prepared and customizable, have potential for the industrial separation of whey proteins.

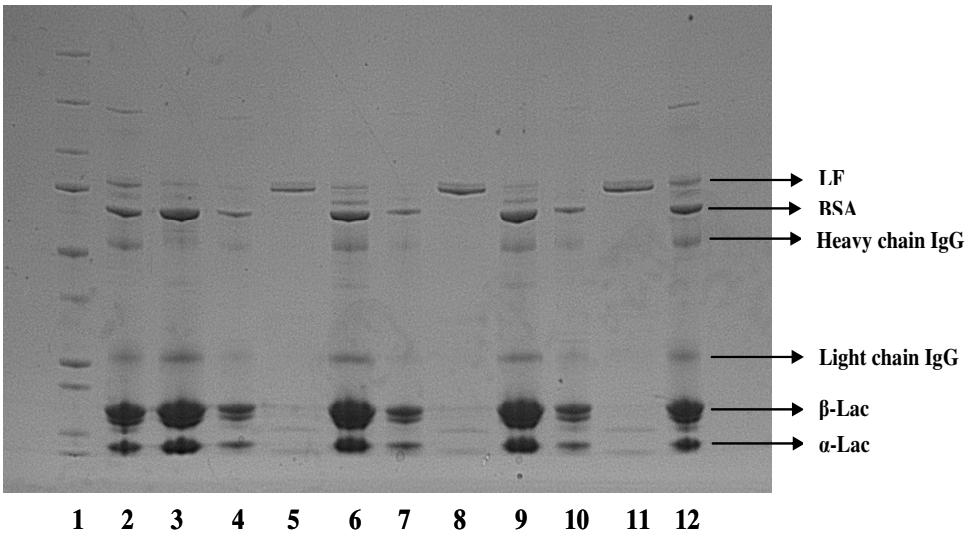


Figure 5-7: SDS-PAGE gel of selected fractions from a cross-flow experiment. Lane 1 - marker; Lane 2 – feed whey. Experiment using one piece of membrane: Lane 3 – whey (retentate) after loading; Lane 4 – permeate from washing step; Lane 5 – permeate from elution step. Experiment using two pieces of membrane: Lane 6 – retentate whey; Lane 7 – permeate washing; Lane 8 – permeate elution. Experiment using three pieces of membrane: Lane 9 – retentate whey; Lane 10 – permeate washing; Lane 11 – permeate elution; Lane 12 – feed whey.

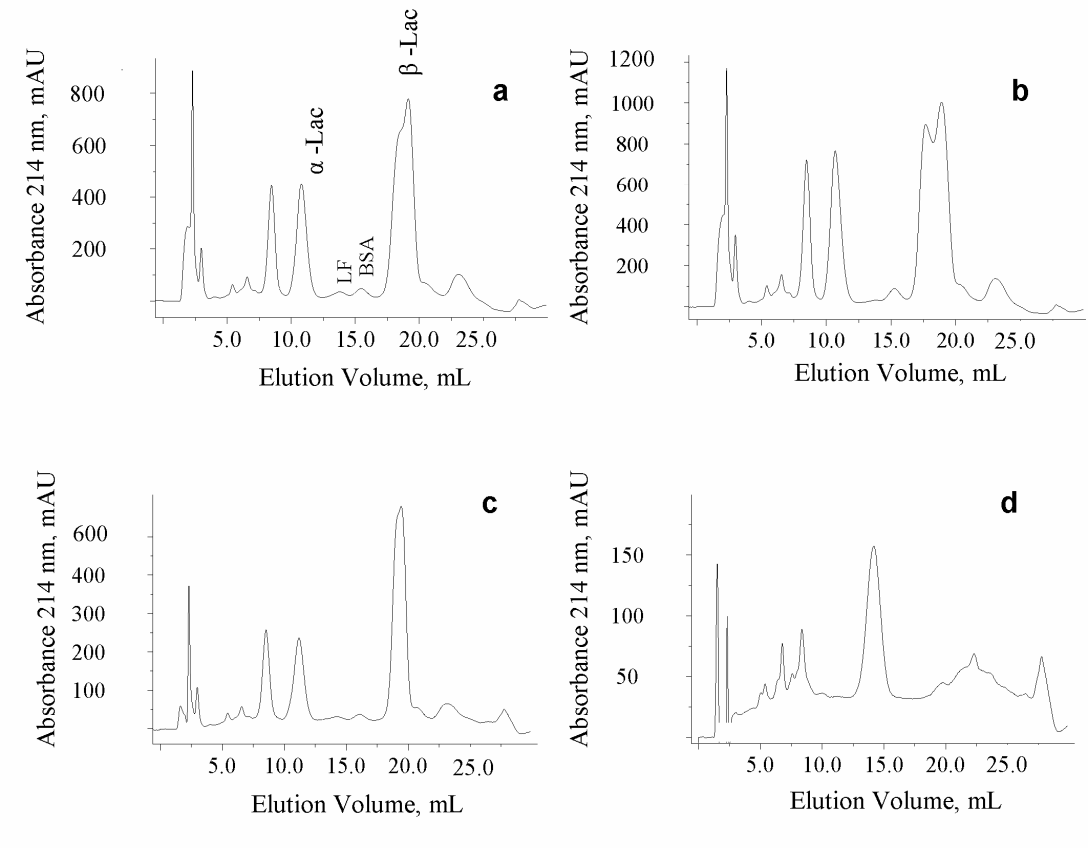


Figure 5-8: Chromatograms for RPC of various solutions in a cross-flow experiment using two pieces of cation exchange MMM for LF recovery from whey. (a) whey – 14X dilution with RPC running buffer (b) retentate whey – 6X dilution with RPC running buffer (c) permeate washing – 2X dilution with RPC running buffer (d) permeate elution – 2X dilution with RPC running buffer. Retention volumes for α-Lac, LF, BSA and β-Lac were approximately 11, 14, 16 and 19 mL, respectively.

6 Phenyl Sepharose hydrophobic interaction mixed matrix membrane chromatography for whey protein fractionation

6.1 Introduction

This chapter describes the development of Phenyl Sepharose hydrophobic interaction MMM chromatography for whey protein fractionation. A flat sheet membrane was fabricated using EVAL base polymer with ground Phenyl Sepharose resin added as an adsorbent particle. The optimum ammonium sulphate concentration in binding buffer was determined for binding the major whey proteins (β-Lac, α-Lac, BSA and LF) at pH 6. The static binding capacity of hydrophobic interaction MMM was measured for the major whey protein components and tested for the feasibility of whey protein fractionation. The performance of hydrophobic interaction MMM was compared with 1 mL HiTrap™ Phenyl FF (GE Healthcare Technologies) for whey protein fractionation.

6.2 Materials and methods

6.2.1 Chemicals

All chemicals, proteins and materials used in this chapter were described previously in section 3.1.

6.2.2 Preparation of mixed matrix membranes

Flat sheet mixed matrix membranes were prepared using a similar technique in section 3.2, but using Phenyl Sepharose resin as the embedded adsorbent. The resin was ground and screened to obtain a particle fraction that passed through a 38 μm stainless steel mesh. Ground resin was added to the prepared polymer solution (15 wt% EVAL and 15 wt% 1-octanol in DMSO) to make a 20 wt% (relative to the EVAL content in the polymer solution) of homogenous casting slurry solution. Conventional dry-wet casting process was then applied to make a flat sheet membrane. The membrane thickness after solvent exchange and drying process was about 200 μm.

6.2.3 Preparation of whey

Whey was prepared according to the method described in section 3.3 and adjusted to pH 6. $(NH_4)_2SO_4$ salt was added into this whey solution to give a salt concentration of 2 M. After the salt was completely dissolved in the solution, whey was centrifuged one more time at 17,902 *g* at 4 °C for 20 min.

6.2.4 Binding capacity of Phenyl Sepharose resin at different ammonium sulphate concentration

About 30 mg (wet mass) of intact Phenyl Sepharose resin was used in salt scouting experiment to determine the optimum salt concentration for binding. $(NH_4)_2SO_4$ salt concentration varied from 0-2.5 M at 0.5 M interval which gives 6 set of experiments. Each experiment was repeated for single protein of α-Lac, β-Lac, BSA and LF.

Pre-equilibrated resin was incubated with 1 mL of 1 mg mL^{-1} of single protein solution in small Eppendorf tubes. Resin and liquid in these tubes were gently mixed by inversion throughout binding for at least 12 h at room temperature, 22°C. After binding, the tubes were spun in a centrifuge at 16,100 *g* for 5 min and the equilibrium protein concentrations in supernatants were assayed by spectrophotometer (as described below). The bound protein was calculated by the difference between the initial protein content to the protein content in the equilibrium solution after binding.

6.2.5 Static binding capacity of adsorbent

Static binding capacity of the adsorbent was measured according to the protocol described in section 3.5. The amount of intact and ground resin used was between 2-4 mg based on dry mass. For membrane, single rectangular piece of membrane with dimensions 12 mm × 22 mm (corresponding to the volume of 5.3×10^{-2} mL) was used with the mass range between 16-20 mg. 2 M $(NH_4)_2SO_4$ in 20 mM sodium phosphate pH 6 was used as a binding buffer. Pre-equilibrated adsorbent was incubated with 1 mL of known concentration protein solution in a series from 0.5 to 5 mg mL^{-1}. Final equilibrium concentration was measured after 12 h incubation period and was assayed by the spectrophotometric method.

6.2.6 Batch elution

In batch elution experiments, three random samples of membrane with a dimension of 15 mm × 15 mm were used. Pre-equilibrated membranes were incubated with 1 mL of 2 mg mL^{-1} protein solution and gently mixed by inversion throughout binding for 12 h. Then, the surface liquid was removed from the membrane by patting with a tissue and the membrane was transferred to a new tube containing elution (salt free) buffer for another 12 h. The concentration of protein in both solutions was assayed and the recovery was calculated as the percentage of protein in the elution fraction per protein bound on the membrane. The elution experiment was also repeated for eluting bound proteins from the range of $(NH_4)_2SO_4$ salt concentrations used in binding buffer.

6.2.7 Flow through experiment

Flow through experiments were conducted on AKTAexplorer™ 100 liquid chromatography system (GE Healthcare Technologies) using Phenyl Sepharose MMM and a 1 mL HiTrap Phenyl FF column. Sheets of MMM were cut into circles of diameter 44 mm to fit into a 47 mm polypropylene filter holder (GE Osmonic Labstore, Minnetonka, MN). The buffer used for flow through experiments was 2 M $(NH_4)_2SO_4$ in 20 mM sodium phosphate pH 6 as a binding buffer and salt free buffer, 20 mM sodium phosphate pH 6 as an elution buffer. The flow rate was kept constant at 3 mL min^{-1} for both columns and the protein absorbance at 280 nm was monitored.

In whey fractionation experiments, the column was equilibrated with 40 mL binding buffer, followed by 8 mL of whey injection, 32 mL washing with binding buffer and isocratic elution with salt free buffer for 40 mL. The flow through fractions were collected at 5 mL fraction volumes for further protein analysis.

6.2.8 Single protein assay

Concentrations of single proteins were determined by measuring absorbance at 280 nm using a UV/Visible spectrophotometer (Model Ultraspec 2100 Pro, Amersham Biosciences, England) as in section 3.7.1.

6.2.9 Whey protein assay

Individual whey protein component was assayed using an established method by Elgar et al. (2000) using 1 mL Resource™ RPC column (GE Healthcare Technologies). Buffer A and buffer B used were 0.1% v/v TFA in DI water and 0.09% v/v TFA, 90% v/v acetonitrile in DI water respectively. The detailed assay protocol was described previously in section 3.8. Samples from flow through experiment were desalted with HiTrap 5 mL desalting column (GE Healthcare Technologies) before being injected onto the RPC column.

6.2.10 Gel electrophoresis

Protein fractions from flow through experiments were analyzed by SDS-PAGE under reducing conditions. The detail protocol was previously described in section 3.9.

6.3 Results and discussion

6.3.1 Ammonium sulphate salt concentration scouting on Phenyl Sepharose resin

Figure 6-1 shows the binding capacity of intact Phenyl Sepharose resin for the single protein components typically found in whey at various $(NH_4)_2SO_4$ concentrations. Although the hydrophobicity of protein normally increases with increasing salt concentration, protein precipitation is another factor that needs to be considered in selecting the optimum salt concentration. At a very high salt concentration, protein tends to precipitate from the solution. The percentage of protein precipitation from 1 mg mL^{-1} protein solution at room temperature, 22°C, in different $(NH_4)_2SO_4$ salt concentrations is shown in figure 6-2. At 1.5 M salt, only β-Lac experienced a small extent of protein precipitation. At 2.0 M salt concentration, all proteins start to precipitate with less than 10% precipitation, except β-Lac which precipitated at about 20%. Severe protein precipitation occurs at salt concentration of 2.5 M, especially for BSA and LF. By considering the resin binding capacity and the protein precipitation percentage, an optimum $(NH_4)_2SO_4$ salt concentration of 2.0 M was selected for further development of Phenyl Sepharose MMM in this study.

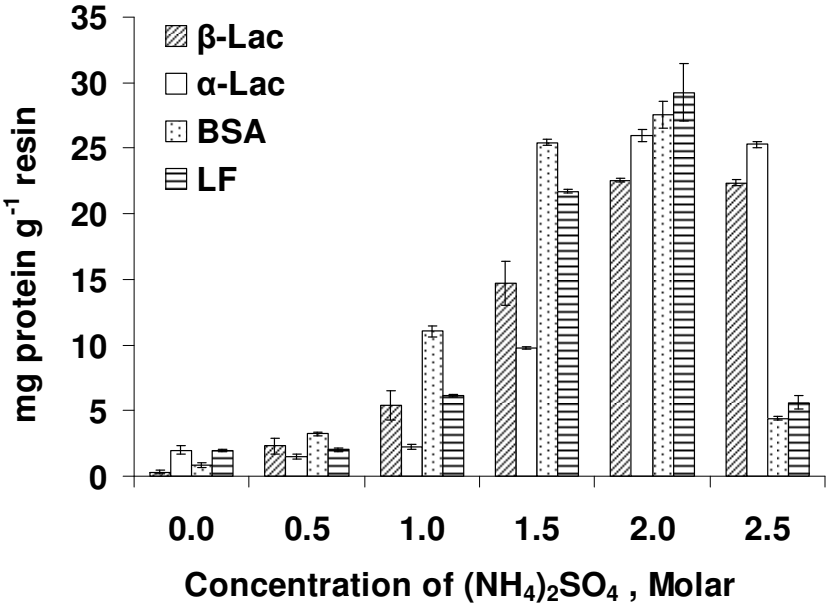


Figure 6-1: Phenyl Sepharose binding capacity at various ammonium sulphate salt concentrations. 1 mg mL^{-1} of single protein solution was incubated with the resin for 12 h at room temperature, 22°C. Error bars are ± one standard deviation (n=3).

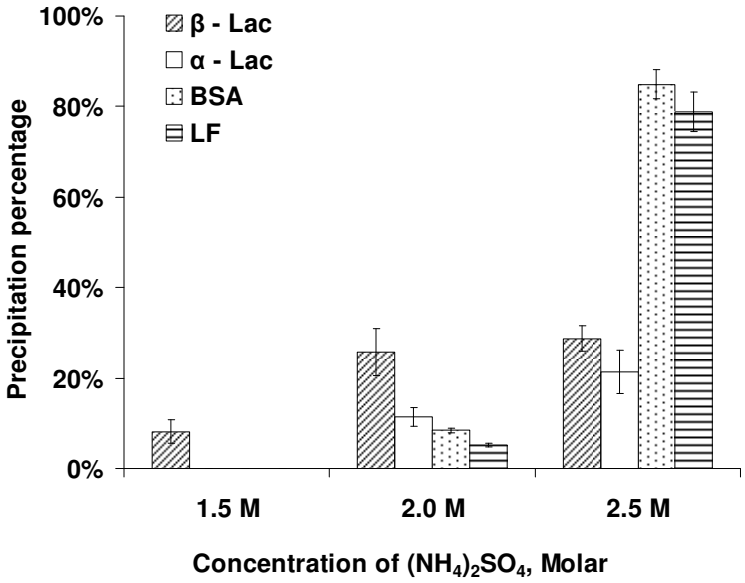


Figure 6-2: Precipitation of 1 mg mL^{-1} of single protein solution at 22°C under gentle mixing by inversion for 12 h in 20 mM sodium phosphate buffer pH 6 at various ammonium sulphate concentrations. Error bars are ± one standard deviation (n=3).

6.3.2 Static binding capacity of intact and ground Phenyl Sepharose resin

The static (equilibrium) binding capacity of intact and ground Phenyl Sepharose resin for single whey protein component is shown in figure 6-3. Both intact and ground resins had a similar protein binding capacity value. The functional group within the pore structure of Phenyl Sepharose resin was fully accessible by the protein even in its intact form. Previously, for anion exchanger resin, Lewatit MP500 in Chapter 4 and cation exchanger resin, SP Sepharose in Chapter 5, grinding improved the protein binding capacity by increasing the resin surface area for binding and enhancing the protein accessibility to the internal pores within the resin. Although the intact Phenyl Sepharose resin already had a good protein accessibility and high binding capacity, grinding into small particles is still necessary to improve the adhesion and homogeneous distribution of the resin within the membrane matrix during the formation of MMM. According to the resin capacity value in figure 6-3, LF has the highest capacity value while β-Lac has the lowest capacity value.

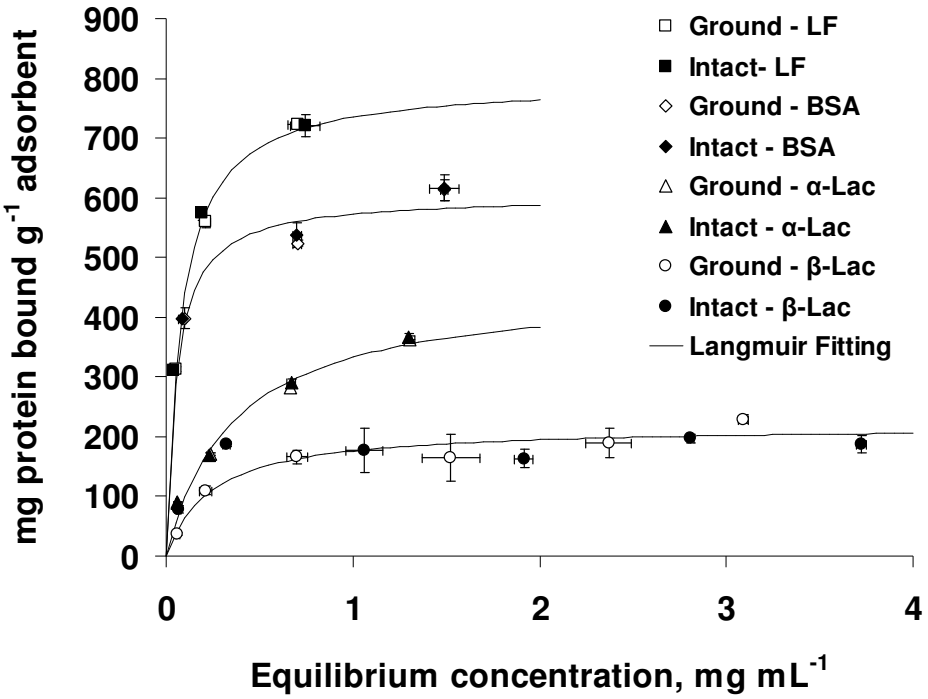


Figure 6-3: Static (equilibrium) binding capacity of intact and ground Phenyl Sepharose resin for single whey protein component in 2 M ammonium sulphate in 20 mM sodium phosphate buffer pH 6. Error bars are ± one standard deviation (n=3).

6.3.3 Static binding capacity of Phenyl Sepharose mixed matrix membrane

The static binding capacity of Phenyl Sepharose MMM is shown in figure 6-4 for single protein components in whey. The data was fitted with the Langmuir isotherm (Equation 3-2). The values for Langmuir isotherm constants are tabulated in table 6-1. Phenyl Sepharose MMM shows a comparative capacity value to that found in the literature: 12 mg mL^{-1} of monoclonal antibody by PVDF membrane (Ghosh 2001), 0.94-2.10 mg cm^{-2} of LZY and 0.78-1.29 mg cm^{-2} of conalbumin by glass fiber membrane modified with short-chain organosilicon (Chen et al. 2007), 30 mg g^{-1} of BSA by butyl amine hollow fiber (Kubota et al. 1997a) and 0.65 mg cm^{-2} of IgG for commercial Sartobind Phenyl membranes (Fraud et al. 2008).

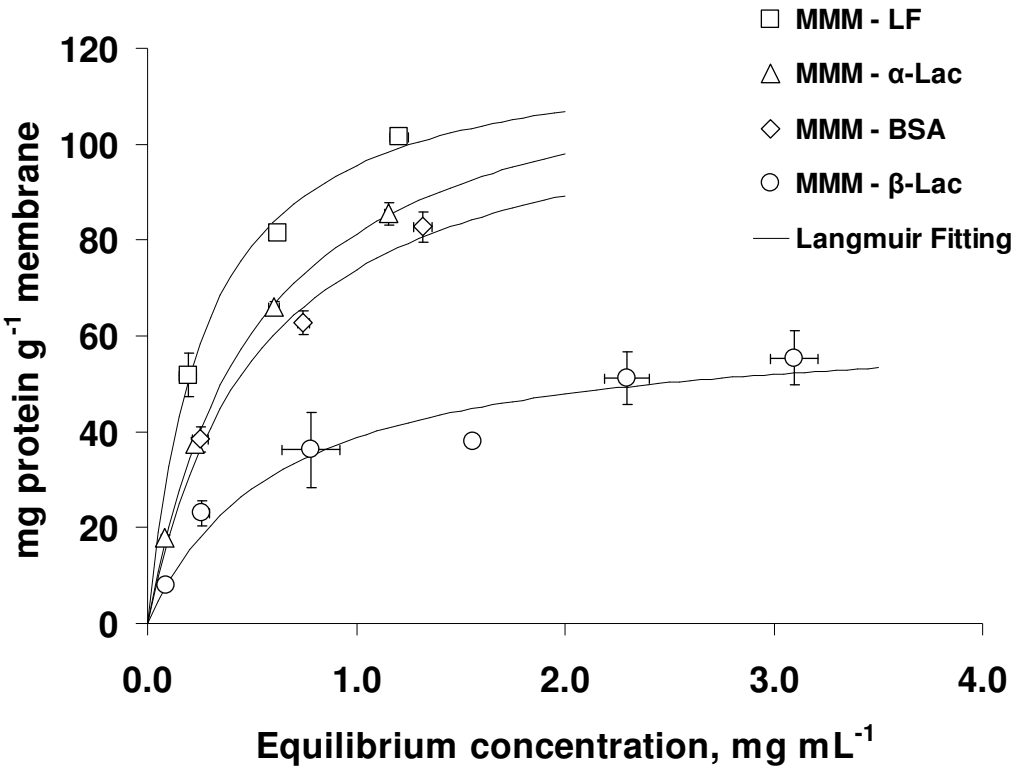


Figure 6-4: Static (equilibrium) binding capacity of Phenyl Sepharose MMM for single whey protein component in 2 M ammonium sulphate in 20 mM sodium phosphate buffer pH 6. Error bars are ± one standard deviation (n=3).

Table 6-1: Langmuir isotherm constant for different proteins by Phenyl Sepharose mixed matrix membrane chromatography.

Langmuir constant	β-Lactoglobulin	α-Lactalbumin	Bovine serum albumin	Lactoferrin
q_m, mg g^{-1}	62.729	123.354	112.930	121.427
K_D, mg g^{-1}	0.620	0.518	0.518	0.271
q_m, mg mL^{-1}	20.539	45.573	38.651	42.046
K_D, mg mL^{-1}	0.475	0.561	0.594	0.382
q_m, mg cm^{-2}	0.411	0.911	0.733	0.841
K_D, mg cm^{-2}	0.475	0.561	0.594	0.382

6.3.4 Batch elution of Phenyl Sepharose mixed matrix membrane

The binding and elution recovery of Phenyl Sepharose MMM and EVAL membrane are shown in figure 6-5. Protein binding only occurs in the presence of $(NH_4)_2SO_4$ salt in the system, as compared to zero salt concentration (figure 6-5(a)) at which there is an insignificant amount of protein bound to the MMM. This suggests that the protein bound onto Phenyl Sepharose MMM is mainly governed by the hydrophobic interaction mechanism. Similar to the optimum salt concentration found in intact Phenyl Sepharose resin, MMM also shows an optimum binding-elution performance at 2 M $(NH_4)_2SO_4$ concentration (figure 6-5 (d)). However, at this salt concentration, the EVAL base membrane also shows some non specific hydrophobic interaction with the protein especially for β-Lac and α-Lac (figure 6-5 (f)). This non-specific binding contributed to higher α-Lac binding capacity than BSA capacity as observed in Phenyl Sepharose MMM (figure 6-4), although the intact and ground resin shows the opposite trend (figure 6-3). The elution recovery of β-Lac was lowest compared to other proteins at 2 M $(NH_4)_2SO_4$ concentration. This may be related to the low elution recovery for some portion of β-Lac bound by non-specific interactions to the EVAL membrane (less than 30% as in figure 6-5 (f)) at 2 M salt.

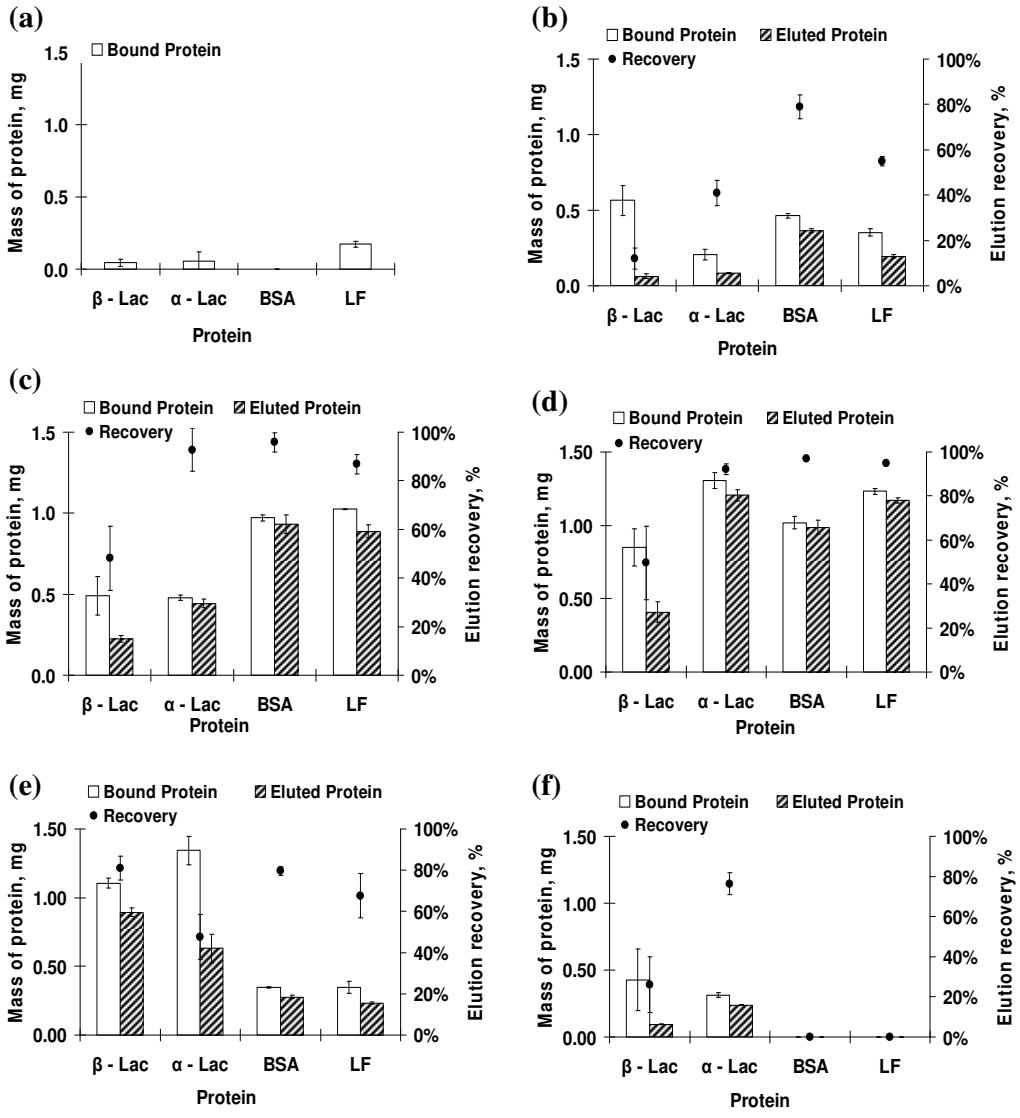


Figure 6-5: Single protein binding of Phenyl Sepharose MMM and EVAL base membrane at different ammonium salt concentration in 20 mM sodium phosphate buffer. 2 mg mL^{-1} of feed protein was prepared in different salt concentration binding buffer and elution was done using salt free buffer. (a) MMM at 0 M salt; (b) MMM at 1.0 M salt; (c) MMM at 1.5 M salt; (d) MMM at 2.0 M salt; (e) MMM at 2.5 M salt and (f) EVAL at 2.0 M salt. Error bars are ± one standard deviation (n=3).

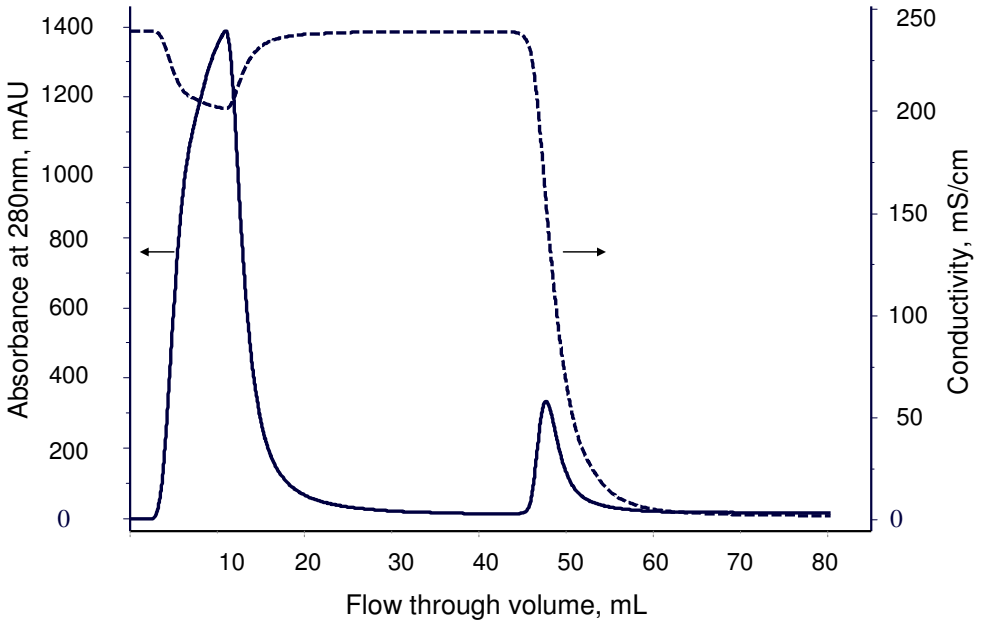
6.3.5 Flow through fraction of whey by Phenyl Sepharose mixed matrix membrane

Whey protein component was precipitated in some extent after the addition of $(NH_4)_2SO_4$ up to 2 M concentration. The precipitation percentage for each protein is shown in table 6-2. The performance of Phenyl Sepharose MMM and HiTrap Phenyl column are compared in table 6-3 for whey protein fractionation in flow through mode. A typical chromatogram for this fractionation is shown in figure 6-6. Although the elution peak of HiTrap Phenyl is larger than Phenyl Sepharose MMM, the performance of Phenyl Sepharose MMM in terms of bound protein per mL of adsorbent used was comparable. According to table 6-3, Phenyl Sepharose MMM shows binding capacities (mg protein bound mL^{-1} column) of 4.728, 1.001, 1.931 and 9.647 for α-Lac, LF, BSA and β-Lac, respectively. For HiTrap Phenyl column, the values are 4.306, 0.572, 1.285 and 11.062 for α-Lac, LF, BSA and β-Lac, respectively.

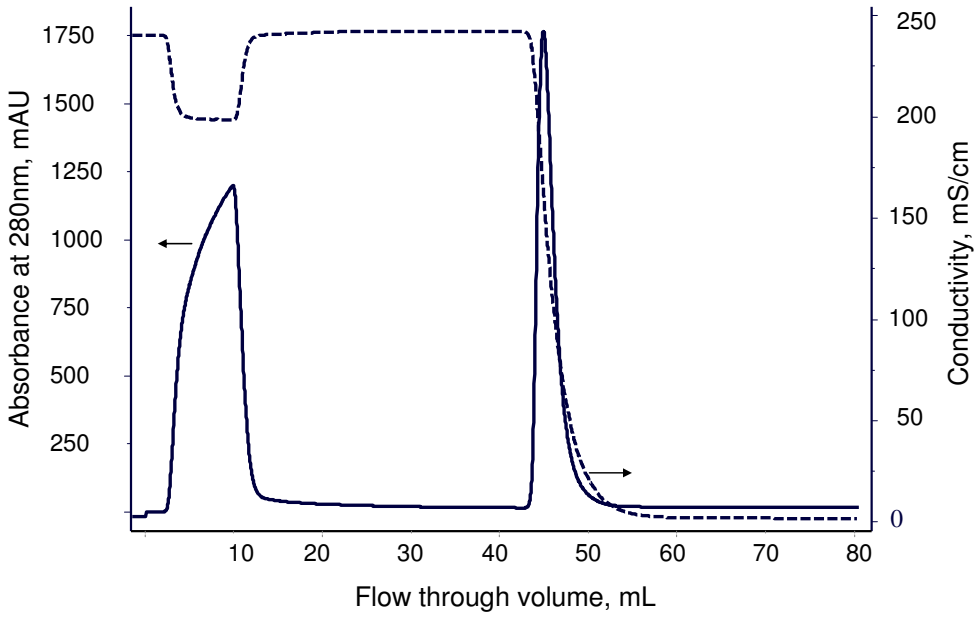
Based on the binding percentage in table 6-3, both columns showed a binding preference for α-Lac, LF and BSA and a lower preference for β-Lac. From the gel picture in figure 6-7, all bound proteins were detected in elution fraction. Compared to commercial HiTrap column, Phenyl Sepharose MMM is suffered from low protein recovery while most of them had an elution recovery less than 80%. This is likely contributed by the non-specific binding of base EVAL membrane at 2 M salt as observed in the previous section.

Table 6-2: Whey protein concentration before and after addition of ammonium sulphate salt at pH 6. After salt addition whey was centrifuged at 17, 902 *g* at 4 °C for 20 min. Data shown is based on average values ± one standard deviation (n=3).

Protein	0 M $(NH_4)_2SO_4$, mg mL^{-1}	2 M $(NH_4)_2SO_4$, mg mL^{-1}	Precipitation percentage, %
α-Lactalbumin	0.897 ± 0.026	0.646 ± 0.015	28
Lactoferrin	0.097 ± 0.015	0.074 ± 0.007	24
Bovine serum albumin	0.195 ± 0.019	0.170 ± 0.024	13
β-Lactoglobulin	3.565 ± 0.106	2.871 ± 0.056	19



(a) Phenyl Sepharose MMM



(b) HiTrap Phenyl column

Figure 6-6: Typical chromatogram for whey fractionation in flow through mode using the AKTAexplorer 100 liquid chromatography system for (a) a Phenyl Sepharose MMM and (b) a HiTrap Phenyl column.

Table 6-3: Flow through fractionation of whey by (a) Phenyl Sepharose mixed matrix membrane chromatography and (b) HiTrap Phenyl 1 mL column at 2 M ammonium sulphate concentration pH 6. Bound protein was eluted in step elution with salt free buffer. Data shown is based on average values ± one standard deviation (n=3).

(a) Phenyl Sepharose mixed matrix membrane

	α-Lac	Lactoferrin	BSA	β-Lac
Feed protein, mg	5.171	0.588	1.364	22.967
Bound protein, mg	1.438 ± 0.095	0.304 ± 0.015	0.587 ± 0.037	2.934 ± 0.357
Binding percentage, %	27.81 ± 1.84	51.75 ± 2.57	43.07 ± 2.68	12.77 ±1.56
mg protein bound mL^{-1} membrane	4.728 ± 0.313	1.001 ±0.050	1.931 ± 0.120	9.647 ± 1.176
Eluted protein, mg	0.995 ± 0.083	0.235 ± 0.019	0.387 ± 0.017	2.214 ± 0.190
Elution recovery, %	69.23 ± 3.52	77.02 ± 3.36	65.88 ± 2.54	76.39 ±13.39

(b) HiTrap Phenyl 1 mL column

	α-Lac	Lactoferrin	BSA	β-Lac
Feed protein, mg	5.171	0.588	1.364	22.967
Bound protein, mg	4.306 ± 0.131	0.572 ± 0.027	1.285 ± 0.041	11.062 ± 0.470
Binding percentage, %	83.27 ± 2.53	97.32 ± 4.64	94.24 ± 3.00	48.17 ± 2.05
mg protein bound mL^{-1} column	4.306 ± 0.131	0.572 ± 0.027	1.285 ± 0.041	11.062 ± 0.470
Eluted protein, mg	4.057 ± 0.184	0.471 ± 0.001	0.955 ± 0.056	10.719 ± 0.736
Elution recovery, %	94.19 ± 1.69	82.40 ± 3.95	74.26 ± 2.63	96.83 ±2.69

Previously, in Chapter 4, an anion exchange MMM showed selective binding for β-Lac in the whey. Therefore, this Phenyl Sepharose MMM can be used as a sequential step to bind unbound fractions from anion exchange MMM or it can also be used alone to produce whey protein isolates which are depleted in β-Lac concentration.

Linear gradient elution was tested for both HiTrap column and Phenyl Sepharose MMM. However, neither column could give a distinctive baseline separation between whey protein components, as illustrated in figure 6-8. Selected fractions from linear gradient elution were further analyzed by SDS-PAGE, as shown in figure 6-9. It was observed that the eluted protein was diluted by the gradient performed, while some protein could not be detected by the gel. This effect was more obvious in Phenyl Sepharose MMM because the column volume was 0.304 mL, compared to a 1 mL column volume for the HiTrap column. Further elution study is therefore necessary to resolve single whey proteins during elution, if possible.

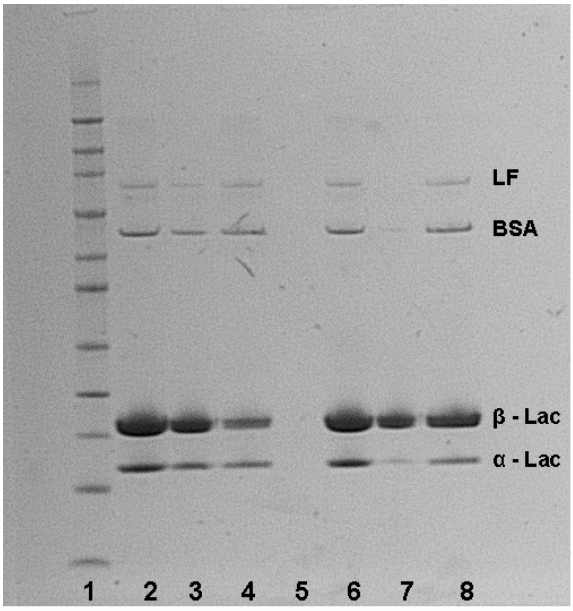


Figure 6-7: SDS-PAGE of whey protein fractions using hydrophobic interaction MMM chromatography: Lane 1 – protein marker, lane 2 – feed whey, lane 3 – unbound fraction, lane 4 – elution fraction, and using Phenyl Sepharose column: lane 6 – feed whey, lane 7 – unbound fraction, lane 8 – elution fraction.

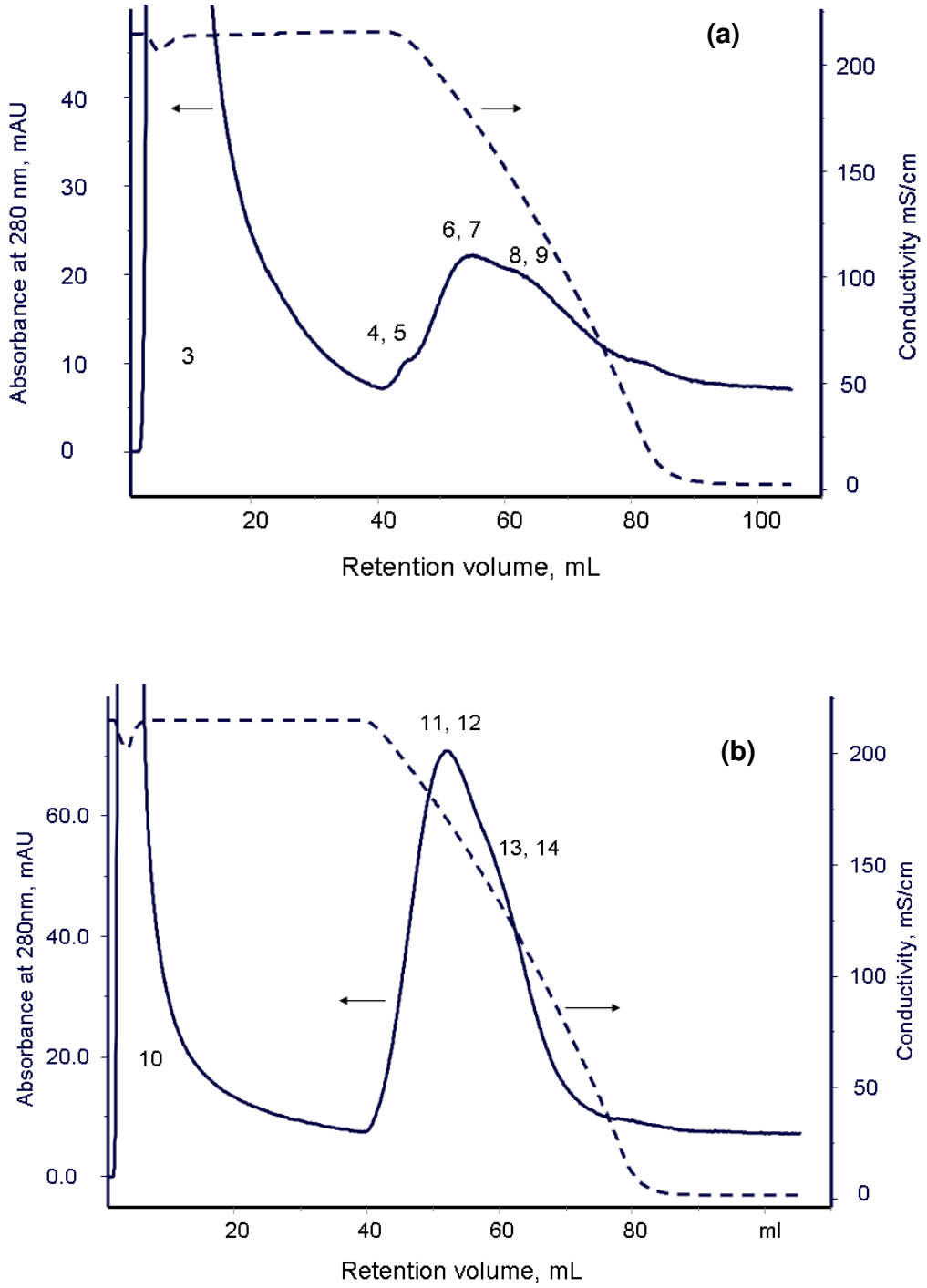


Figure 6-8: Linear gradient elution from a hydrophobic interaction column for whey protein fractionation. 2 mL of whey was loaded onto (a) a 0.304 mL Phenyl Sepharose mixed matrix membrane chromatography column and (b) a 1 mL HiTrap Phenyl Sepharose column.

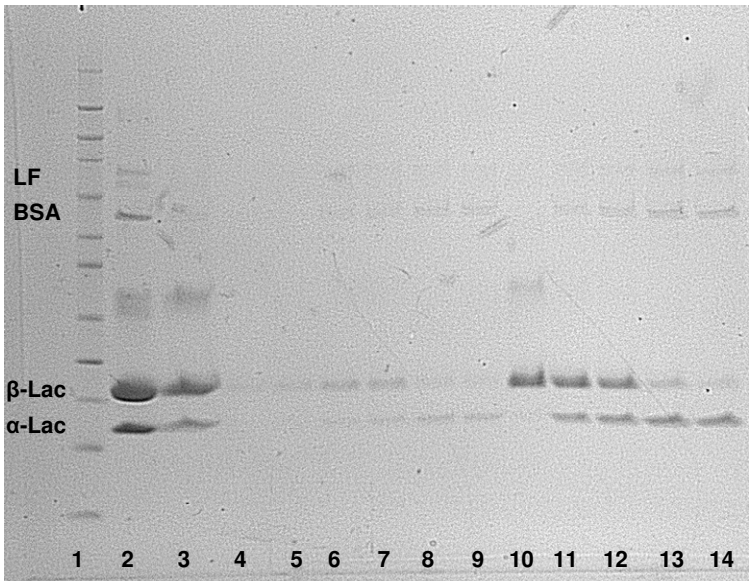


Figure 6-9: Fractions from gradient elution of whey protein using a hydrophobic interaction column. The lane number corresponds to the fractions in figure 6-8. Lanes 1 and 2 represent protein markers and feed whey, respectively.

6.4 Conclusions

Mixed matrix membrane concept has successfully been applied to make anion or cation exchanger membrane chromatography in the previous studies. For the first time, the current study has demonstrated the feasibility of incorporating hydrophobic Phenyl Sepharose resin into a membrane matrix to prepare hydrophobic interaction membrane chromatography. Phenyl Sepharose MMM chromatography was tested for fractionation of whey protein components at 2 M ammonium sulphate salt concentration. The performance of Phenyl Sepharose MMM was comparable with a 1 mL HiTrap Phenyl column. Phenyl Sepharose MMM shows binding capacities (mg protein bound mL^{-1} column) of 4.728, 1.001, 1.931 and 9.647 for α-Lac, LF, BSA and β-Lac, respectively. Both columns show a lower binding percentage of β-Lac, which can be useful in producing whey protein isolates depleted in β-Lac. Furthermore, this Phenyl Sepharose MMM can potentially be used in isolating high purity α-Lac from whey protein, especially if some of the calcium is chelated out, for example using EDTA.

7 Mixed mode interactions in mixed matrix membrane chromatography for protein separation

7.1 Introduction

In the present chapter, the feasibility of using the MMM chromatography preparation concept was demonstrated for producing a novel mixed mode interaction membrane in a single membrane. Mixed mode interaction MMM was synthesized by incorporating a certain ratio of SP Sepharose cation resin and Lewatit MP500 anion resin into an EVAL base polymer solution. The static binding capacity of mixed mode MMM was measured and compared with pure cationic and anionic MMMs. The membrane was also tested for binding to a range of model protein mixtures and whey protein solutions. Different elution protocols were investigated to elute the bound protein from the mixed mode MMM. The productivity of mixed mode MMM chromatography for recovering all major proteins in whey was predicted from flow through experimental data. This mixed mode interaction membrane chromatography has not been studied before and to the best of the author's knowledge this development is the first of its kind to date.

7.2 Materials and methods

7.2.1 Materials

All chemicals, proteins and materials used in this chapter were described previously in section 3.1.

7.2.2 Mixed mode membrane preparation

Base membrane polymer solution was prepared according to section 3.2 previously which consisted of 15 wt% EVAL polymer dissolved in 15 wt% of 1-octanol and 70 wt% of DMSO. Relative to the mass of EVAL in the polymer solution, a different ratio of anion exchanger resin, Lewatit MP500 and cation exchanger resin, SP Sepharose was added to this solution and mixed homogenously to form a membrane casting solution.

Two different compositions of mixed mode interaction membrane were prepared. The first casting solution consisted of equal amounts of anion and cation resins, 15 wt% of MP500 and 15 wt% of SP Sepharose ("MMM 1"). The second solution was set to have a total resin loading of 50 wt% and targeted for capturing all proteins in whey. Based on the major protein composition in whey, almost 95% are acidic proteins (β-Lac, BSA, α-Lac, IgG) (table 2-1) and the rest are basic proteins (LF, LP, IgG). This would indicate that a mixed mode membrane, based on these proportions, should comprise 95% anion and 5% cation exchange resins, ignoring the relative protein binding capacities of each type. However, for a more practical membrane casting formulation, the ratio of 85% acidic protein and 15% basic protein was used, which produced a mixed mode membrane with 42.5 wt% of MP500 and 7.5 wt% SP Sepharose ("MMM 2"). The assumption is that the membrane should be operated well below its total equilibrium protein binding capacity so the 85% proportion of anion exchange resin should be more than sufficient, while the 15% cationic resin will almost certainly bind the maximum basic proteins as long as the anionic fraction is not overloaded.

A conventional casting method was used for preparing a flat sheet membrane as described in section 3.2. The thickness of the membrane after drying was about 200 μm.

7.2.3 Protein binding capacity

A small piece of membrane with 15 mm × 15 mm dimension (mass around 8-13 mg, volume 4.5×10^{-2} mL) was used for protein binding experiment. 20 mM sodium phosphate, pH 6 was used an equilibrium/binding buffer. All membranes were pre-equilibrated with equilibrium buffer for at least 3 h or more before binding to any known concentration of protein solution. At least triplicate samples were used for each experiment.

For determination of the static binding capacity of mixed mode MMM 1, a pre-equilibrated membrane was incubated with 1 mL of pure β-Lac or LZY protein solutions with different initial concentrations ranging from 0.25-4.00 mg mL^{-1}. The equilibrium protein concentration was assayed after 12 h binding time. The capacity-

equilibrium data were fitted to the Langmuir isotherm using the least-square regression method described in section 3.5.

Two acidic proteins (β-Lac, BSA) and two basic proteins (cytochrome C, LZY) were selected as model proteins in a quaternary protein mixture for binding to MMM 1. The level of protein concentration in the mixture was varied for both acidic and basic proteins either as low (0.5 mg mL^{-1}), medium (2.0 mg mL^{-1}) or high (4.0 mg mL^{-1}) concentrations. In some cases, the membrane was also tested for binding to a ternary proteins mixture of β-Lac, BSA and LZY, or binary mixtures of β-Lac and BSA or LF and β-Lac. The protein concentrations were assayed using reversed phase chromatography as described below.

7.2.4 Elution experiment

7.2.4.1 Batch elution

MMM 1 was tested for binding and eluting of pure protein solutions of 1 mg mL^{-1} β-Lac and 1 mg mL^{-1} LZY, and a binary protein mixture of β-Lac and LZY with concentration of 1 mg mL^{-1} for each protein. A similar binding procedure was followed as described above. After the binding step, the membrane was dried by lightly patting with tissue and underwent a washing step with binding buffer for 30 minutes. The membrane was removed from the washing solution and the liquid on the surface of the membrane was again dried by patting. 1 M NaCl in binding buffer was used as an elution buffer and added to the membrane for at least 12 h elution time. Throughout the experiment, 1 mL of solution was used at each step.

7.2.4.2 Flow through elution

Flow through experiments were conducted using one piece of membrane 44 mm in diameter, inserted into a polypropylene filter holder (GE Osmonics Labstore, Minnetonka, MN, USA). The module was connected to an AKTAexplorer 100 (GE Healthcare Technologies) liquid chromatography system. Pure cation exchanger membrane (CEX MMM), pure anion exchanger membrane (AEX MMM) and mixed mode MMM 1 were tested for flow through elution. Pure protein solutions of β-Lac or LZY and a binary protein solution of β-Lac and LZY were loaded onto the

membrane. The concentration of each protein was 1 mg mL^{-1} and a flow rate of 3 mL min^{-1} was used throughout the flow through experiment.

Two elutions protocols were tested: (1) isocratic elution with salt and (2) pH elution, followed by salt elution. In the isocratic salt elution protocol, the membrane was equilibrated with 35 mL of 20 mM sodium phosphate pH 6 (binding buffer), injected with 2 mL of protein sample, followed by 23 mL of washing with binding buffer and finally eluted isocratically with 1 M NaCl in the binding buffer for 25 mL. In the pH elution protocol, the same procedure was followed excepted using two elution steps: 25 mL of 100 mM sodium acetate pH 4 was followed by a second elution with 1 M NaCl in the binding buffer for another 35 mL. 2 mL flow through fractions were collected in each step by an automatic fraction collector, Frac-950 (GE Healthcare Technologies).

7.2.5 Whey fractionation

7.2.5.1 Batch fractionation

Throughout the whey fractionation experiment, MMM 2 was used. In batch binding of whey, the same protocol as that described in section 7.2.4.1 was followed except the dimensions of the membrane used was 12 mm × 22 mm. Whey was prepared according to section 3.3 and spiked with an additional amount of LF. Both normal whey and LF-spiked whey were tested for batch fractionation.

7.2.5.2 Cross-flow fractionation

Whey fractionation in cross-flow filtration mode was conducted in the AKTAcrossflow (GE Healthcare Technologies) system using one piece of MMM 2 (membrane area 50 cm^2). A detailed description of the cross-flow system was given previously in section 3.10. 30 mL of LF-spiked whey was used as feed. The membrane was pre-treated with 0.5 M NaOH for 30 minutes and flushed with water until a neutral pH was obtained before running each cross-flow whey fractionation experiment. Feed flow rate and permeate flux were kept constant at 50 mL min^{-1} and 100 LMH, respectively. During the whey loading step, both retentate and permeate stream were recycled to the reservoir until a cumulative permeate volume of 150 mL

was reached. Bound protein was eluted with 1 M NaCl in 20 mM sodium phosphate pH 6. Cross-flow runs were repeated three times with a fresh membrane used in each run.

7.2.5.3 Flow through fractionation

Flow through fractionation was used to determine the capability of MMM 2 to extract all whey protein in a single run. Different volumes of LF-spiked whey were loaded into three layers of membrane in the filter holder module. The setup was similar to that described in section 7.2.4.2 but a flow rate of 2 mL min^{-1} was applied. Different elution protocols were also studied for MMM 2 after 1 mL of LF-spiked whey was injected onto it.

7.2.6 Protein assay

All single or mixture protein concentrations were assayed using a 1 mL Resource RPC (GE Healthcare Technologies). The column was operated on an AKTAexplorer 10 liquid chromatography system controlled by Unicorn 4.0 software (GE Healthcare Technologies). Buffer A and buffer B used were 0.1% v/v TFA in DI water and 0.09% v/v TFA, 90% v/v acetonitrile in DI water respectively. Throughout the analysis experiment, a flow rate of 2 mL min^{-1} was used. Different assay protocols were used depending on the protein mixture, as described in the following section.

7.2.6.1 Quaternary protein mixture

A standard curve for the quaternary mixture of β-Lac, BSA, cytochrome C and LZY was developed from a mixture of the individual proteins. The protein absorbance at 280 nm was monitored during the assay protocol. After 4 CV of column equilibration with buffer A, 500 µL of sample was manually injected into the column. A series of linear gradients was then applied as follows: 0-2 CV, 0% B; 2-7 CV, 0-35% B; 7-13 CV, 35-65% B. After the last gradient, the column was hold at 100% B for 3 CV and followed by 1 CV of re-equilibration with buffer A. Figure 7-1 shows a chromatogram for the quaternary protein mixture and its respective individual proteins obtained in RPC assay.

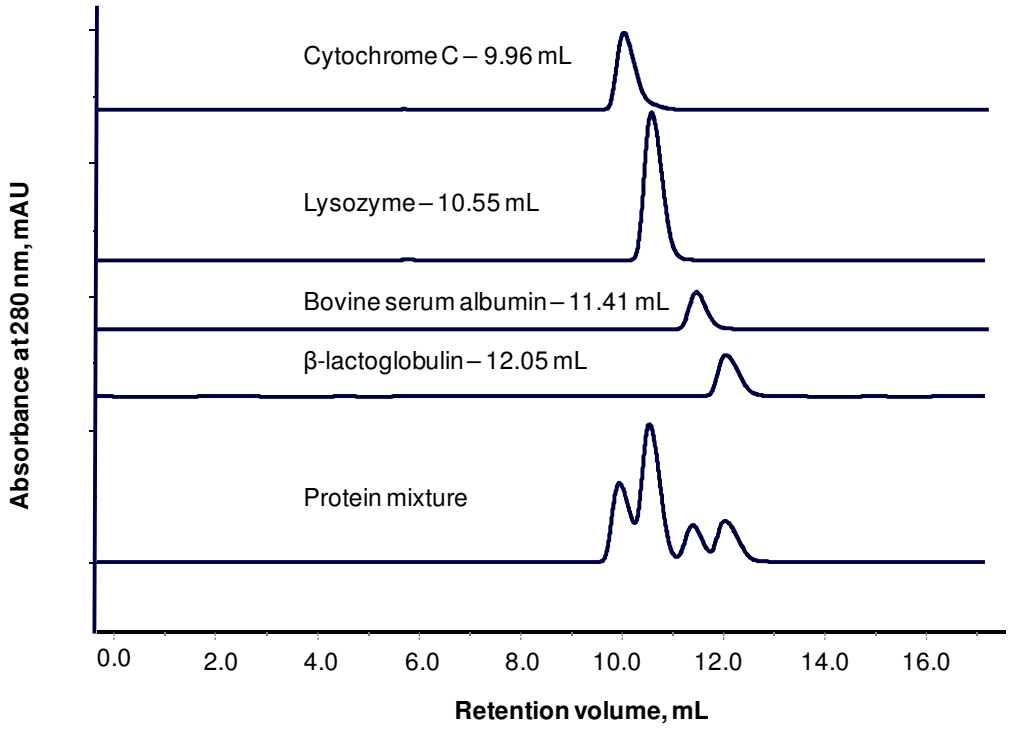


Figure 7-1: Chromatogram for quaternary protein mixture and its respective individual proteins assayed by 1 mL Resource RPC column.

7.2.6.2 Binary β-lactoglobulin and lactoferrin

A similar assay protocol as in quaternary protein mixture was used to quantify the individual protein content in binary the β-Lac-LF solution. However, a separate standard curve was developed because LF was eluted at a similar retention volume to BSA by this protocol.

7.2.6.3 Whey protein assay

Whey's protein components were assayed using an established method by Elgar et al. (Elgar et al. 2000; Palmano and Elgar 2002). Protein absorbance at 214 mAU was used to calculate the peak area to quantify the protein content in whey. The protocol was run at flow rate 2 mL min^{-1} with 100 μL of sample injected manually into the column. The detailed protocol was described in section 3.8.

7.2.7 Gel electrophoresis

Protein fractions from the experiment were analyzed by SDS-PAGE under non-reducing conditions. The detailed protocol was previously described in section 3.9.

7.3 Results and discussion

7.3.1 Static binding capacity of SP Sepharose based adsorbent

Figure 7-2 shows the static binding capacity of SP Sepharose based adsorbent for pure LZY in a single mode CEX MMM (30 wt% of cation resin relative to polymer content, prepared in previously in Chapter 5 and MMM 1 (15 wt% of cation resin, 15 wt% anion resin). The LZY binding capacity was normalized to the content of SP Sepharose resin in the membranes in order to compare their capacities with ground SP Sepharose resin. Based on the normalized resin capacity, some portion of the SP Sepharose resin was apparently not accessible by the protein in the pure CEX MMM. As discussed in Chapter 5, SP Sepharose resin swelled strongly in the solvent used in preparing the membrane casting solutions and resulted in a relatively high viscosity of the casting solution. This viscosity factor may be responsible for the blockage of some part of the active groups in the SP Sepharose resin. For the mixed mode interaction membrane, in addition to some portion of active group blockage, the existence of anion resin in the membrane matrix may interfere with the binding capability of the cation resin to some extent. Both anion resin and LZY molecules were positively charged and repelled each other but this is not expected to be a very strong effect because SP Sepharose has a strong binding to LZY.

7.3.2 Static binding capacity of MP500 based adsorbent

Figure 7-3 shows the anion exchanger resin, MP500 in a pure AEX MMM (50 wt% anion resin, prepared in Chapter 4 was fully accessible by the protein when normalized to the resin content. However, in the MMM 1 (15 wt% anion resin, 15 wt% cation resin), the accessibility of β-Lac to the resin has been affected to a small extent by the presence of the cation resin, especially at the highest protein concentrations. The viscosity of MP500 casting solution is relatively low compared to SP Sepharose resin and the problem of the active group blocking by the polymer

was absent. In a previous preparation, as high as 65 wt% of MP500 resin could be incorporated into MMM (Avramescu et al. 2003a).

7.3.3 Protein binding to the mixed mode MMM

Acidic proteins (β-Lac and BSA) and basic proteins (Cytochrome C and LZY) were mixed together for binding to the mixed mode MMM 1 at different initial protein concentrations. Figure 7-4 shows the membrane capacity plotted with increasing initial basic protein concentration on the x-axis. Figure 7-5, uses the same data as Figure 7-4 but it is arranged so that the x-axis represents the initial acidic protein concentration. In both figures, mixed mode MMM 1 had a preferential binding capacity for β-Lac and LZY compared with BSA and cytochrome C.

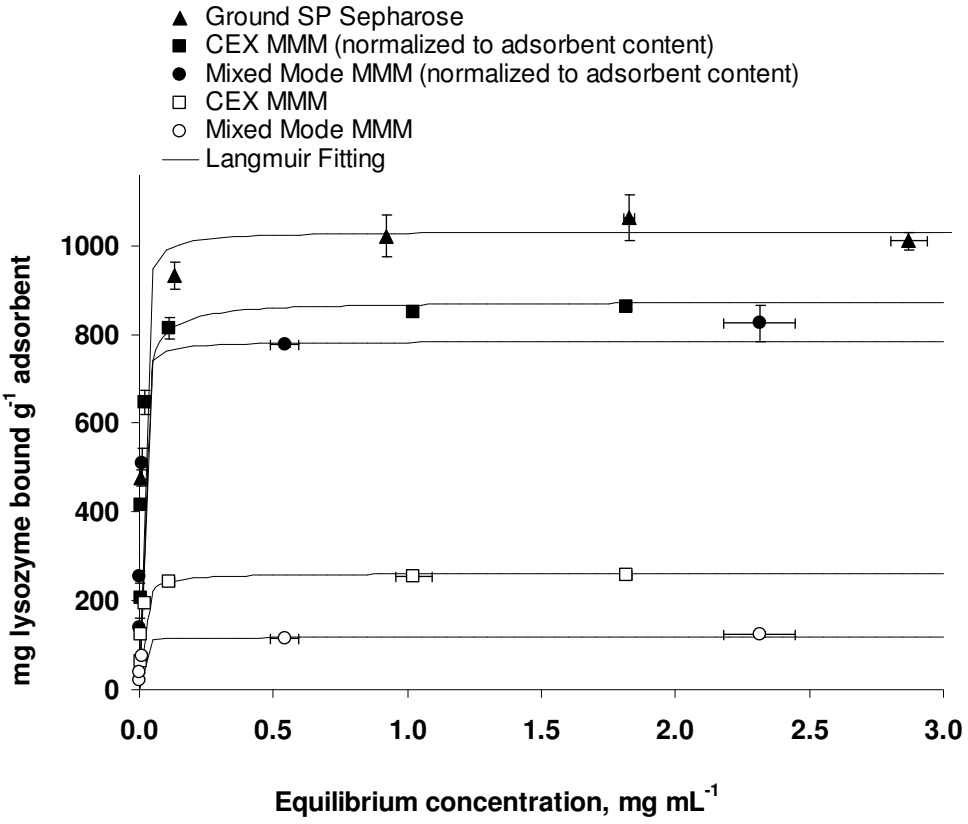


Figure 7-2: Static binding capacity of SP Sepharose based adsorbent for pure LZY in ground resin, a cation exchange membrane (CEX MMM) and mixed mode interaction membrane (MMM 1). Error bars are ± one standard deviation (n=3).

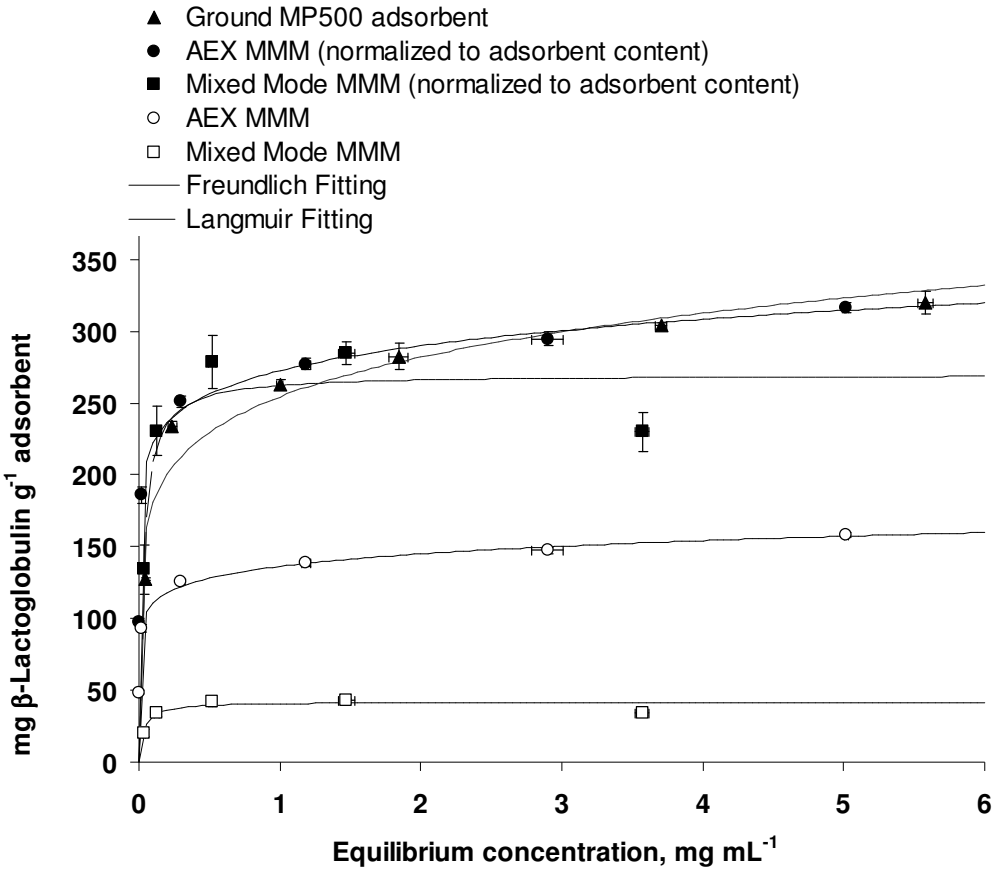


Figure 7-3: Static binding capacity of MP500 based adsorbent for pure β-Lac in ground resin, a pure anion exchanger membrane (AEX MMM) and a mixed mode interaction membrane (MMM 1).

As in figure 7-4, when the initial basic protein concentration increased, the LZY binding capacity increased accordingly. Almost no cytochrome C bound at high or medium LZY concentrations used. In addition, the LZY capacity was not influenced by the concentration level of acidic proteins in the protein solution. However, for acidic protein binding, as we can see in figures 7-4 (c) and 7-4 (e), representing medium and high initial acidic protein concentration respectively, the β-Lac binding dropped at low (0.5 mg mL^{-1}) initial basic protein concentrations.

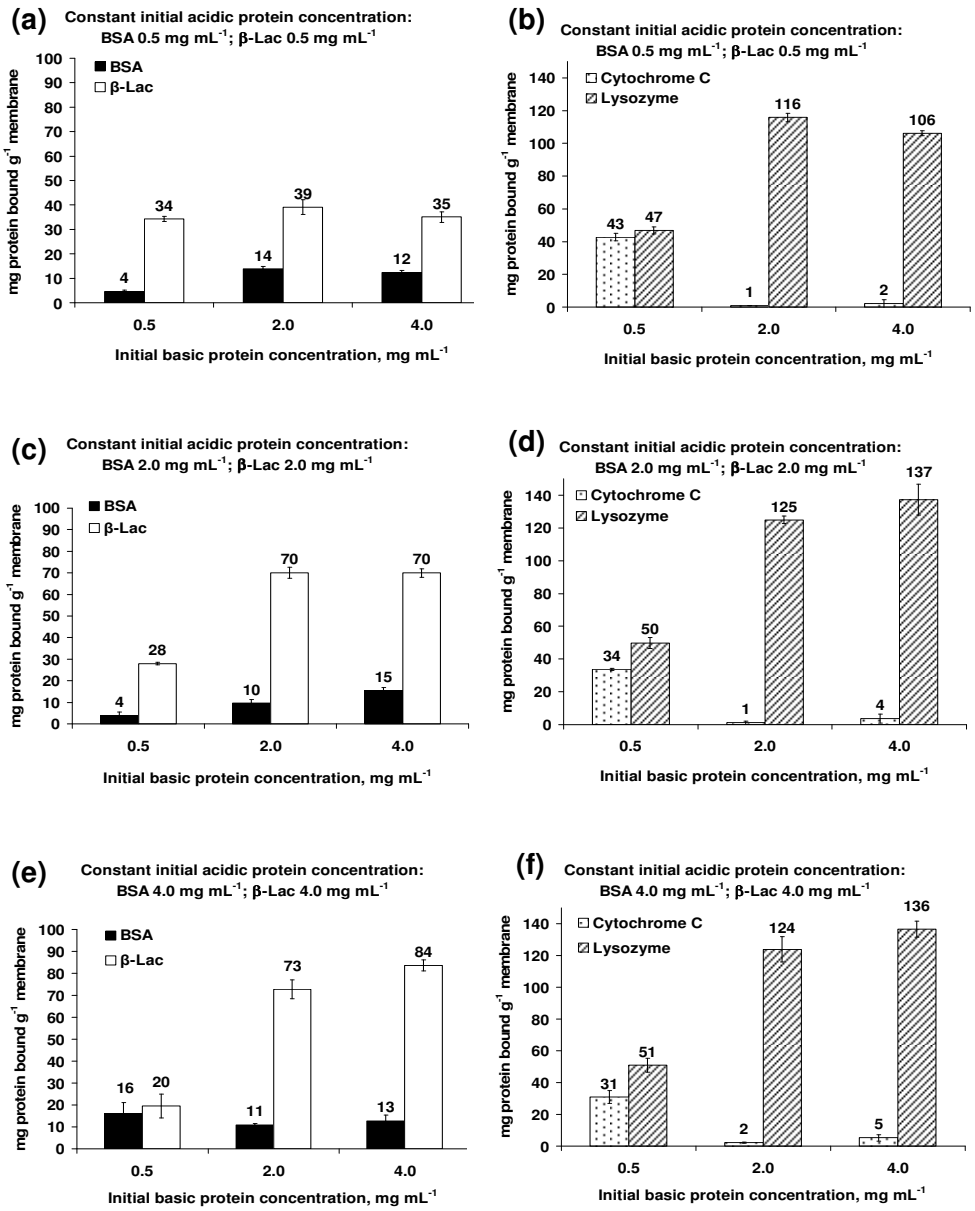


Figure 7-4: Protein binding capacity of mixed mode MMM 1 for quaternary protein mixtures at different initial acidic and basic protein concentrations. The initial concentrations of BSA and β-Lac were fixed at 0.5 mg mL^{-1} (a), (b), 2.0 mg mL^{-1} (c), (d) and 4.0 mg mL^{-1} (e), (f). The binding capacity for acidic proteins (BSA and β-Lac) are represented in (a), (c) and (e) and for basic proteins (cytochrome C and lysozyme) in (b), (d) and (f). The x-axis represents the initial basic protein concentration. Error bars are ± one standard deviation (n=3).

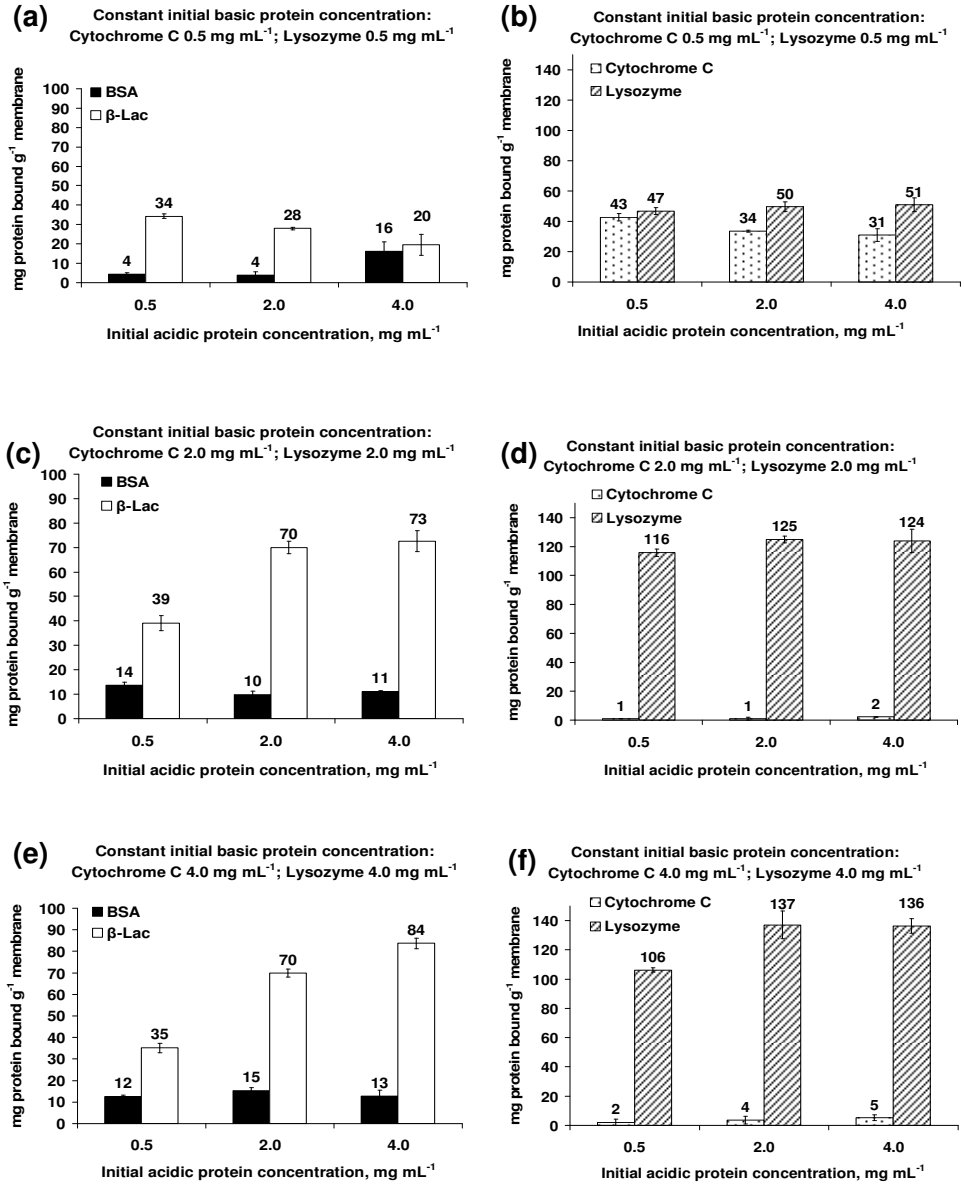


Figure 7-5: Protein binding capacity of mixed mode MMM 1 for quaternary protein mixtures at different initial acidic and basic protein concentrations. The initial concentration for cytochrome C and lysozyme were fixed at 0.5 mg mL^{-1} (a), (b), 2.0 mg mL^{-1} (c), (d) and 4.0 mg mL^{-1} (e), (f). The binding capacity for acidic proteins (BSA and β-Lac) are represented in (a), (c) and (e) and for the basic proteins (cytochrome C and lysozyme) in (b), (d) and (f). The x-axis represents the initial acidic protein concentration. Error bars are ± one standard deviation (n=3).

In order to examine this trend more clearly, the way data presentation was re-arranged to show the initial acidic protein concentration on the x-axis in figure 7-5. From figure 7-5 (a), it is clear that, at low basic protein concentration, an increased in acidic protein concentration does not increase the acidic protein binding capacity proportionately. As a first thought, it was suspected that the existence of cytochrome C on the membrane influenced acidic protein binding. At low initial basic protein concentrations, more cytochrome C was bound, because excess cation groups were still available in the membrane after almost all LZY was bound (figure 7-5 (b)). To justify this hypothesis, the membrane was tested for binding from the ternary protein mixture, in which cytochrome C was excluded, at a low LZY concentration and varying β-Lac and BSA protein concentrations. The binding trend is plotted in figure 7-6.

As shown in figure 7-6, the β-Lac binding capacity still did not increase according to the increase in acidic protein concentration despite the absence of cytochrome C. Therefore, the β-Lac binding was not influenced by the existence of cytochrome C on the membrane. Another possibility might be the free cation group left after binding with a low basic protein concentration would repel the acidic proteins from approaching the anion groups. However, this is unlikely to be the case because the static binding capacity for pure β-Lac was similar to that of the ground resin when the protein capacity was normalized to the resin content in the membrane. To confirm this, a binary β-Lac and BSA mixture was used as a protein solution and the resulting binding capacity is shown in figure 7-7.

Similar to previous observations, the β-Lac binding capacity still did not increase with an increase in the initial concentration of this protein in the protein solution. However, when doing ternary and binary protein experiments, it was noticed that the solution of the ternary protein system was slightly cloudy compared to the binary β-Lac-BSA protein system. This indicates that some interaction occurred between β-Lac and LZY in the solution that might cause precipitation. This was more obvious when a high β-Lac concentration was used. If this precipitation happened during experiments, the β-Lac protein binding in the quaternary protein system in figure 7-4 (c) and figure 7-4 (e) would have been over calculated (because capacity was

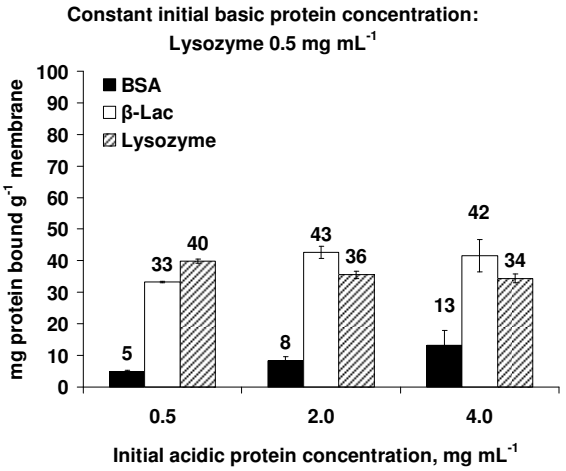


Figure 7-6: Protein binding capacity of mixed mode MMM 1 to ternary protein system of BSA, β-Lac and LZY. Error bars are ± one standard deviation (n=3).

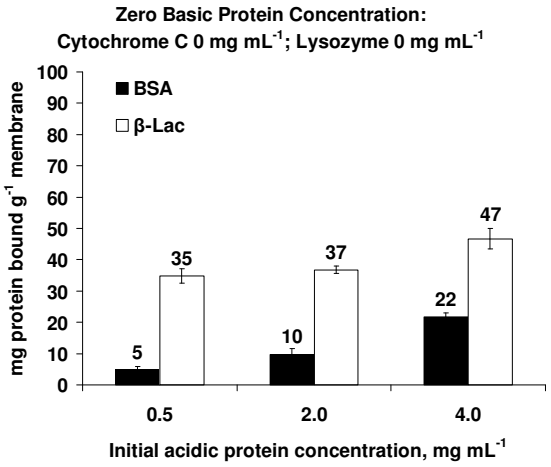


Figure 7-7: Protein binding capacity of mixed mode MMM 1 to a binary protein system of BSA and β-Lac. Error bars are ± one standard deviation (n=3).

calculated based on the equilibrium protein concentration after binding). Furthermore, this cloudiness was not easily noticed initially in experiments with the quaternary mixture because the presence of cytochrome C would change the clear protein solution to a red color. Based on this observation, the normalized β-Lac binding capacity in figure 7-5 (a) was actually already close to its maximum value for the small area of membrane used. If the average β-Lac capacity was around 30 mg β-Lac g^{-1} MMM in figures 7-6 and 7-7, the normalized value would correspond to 200 mg β-Lac g^{-1} MP500. This value is very close to the static capacity of β-Lac shown in figure 7-2.

In further experiments, LZY was replaced by LF to form another binary protein solution with β-Lac to avoid β-Lac interaction with the basic protein in the solution. The protein binding capacity for this new binary protein solution is shown in figure 7-8. It is clear from this that the anion exchanger had reached its capacity for the small area of membrane used and β-Lac binding could not be further increased at higher initial β-Lac concentrations unless a larger membrane area was used. For LF, increasing the LF protein concentration did increase the protein binding proportionately because the cation resin capacity still had not reached its maximum value (based on the LZY capacity in figure 7-3, the ground cation resin would have a maximum capacity of around 1000 mg LZY g^{-1} SP Sepharose). As a conclusion, mixed mode ionic interaction in a single membrane matrix was successfully demonstrated to bind both acidic and basic proteins simultaneously from a mixed protein solution.

7.3.4 Protein elution

Batch elution data are shown in table 7-1 for MMM 1 for pure β-Lac, pure LZY and the binary β-Lac and LZY mixture. The capacity of the membrane for both proteins is close to the value previously reported in the Langmuir plot of static binding capacity in figures 7-2 and 7-3 (the values at equilibrium concentration being less than 0.5 mg mL^{-1} for β-Lac and less than 0.05 mg mL^{-1} for LZY). The elution recovery for LZY was higher than for β-Lac in static elution data.

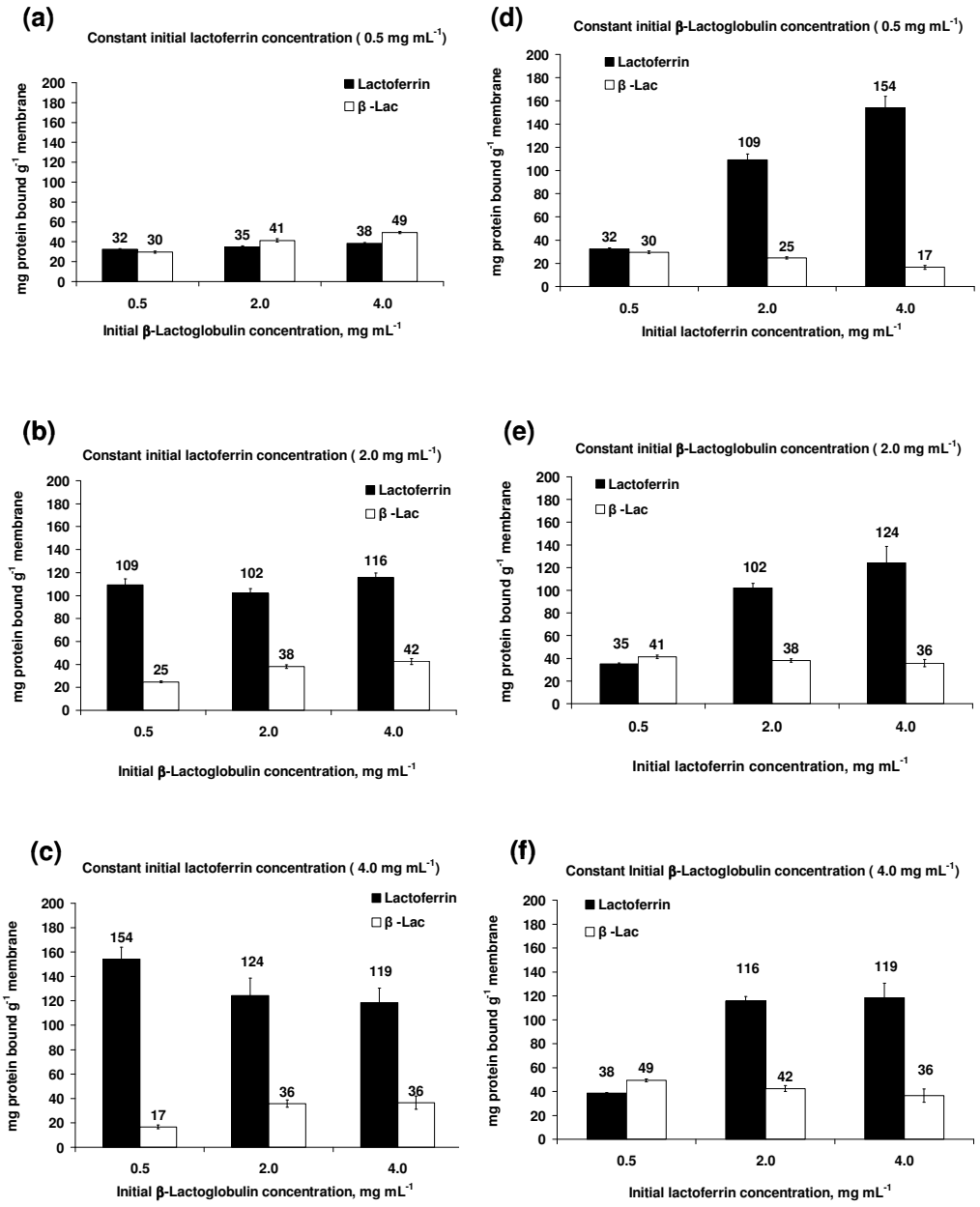


Figure 7-8: Protein binding capacity of mixed mode MMM 1 for the binary protein system of β-Lac and LF at various initial concentrations. One set of experiment was conducted with a constant initial lactoferrin concentration at (a) 0.5 mg mL^{-1}, (b) 2.0 mg mL^{-1} and (c) 4.0 mg mL^{-1}, while the concentration of β-lactoglobulin was varied from 0.5 to 4.0 mg mL^{-1}. Another set of experiments was conducted with a constant initial β-lactoglobulin concentration at (a) 0.5 mg mL^{-1}, (b) 2.0 mg mL^{-1} and (c) 4.0 mg mL^{-1}, while the concentration of lactoferrin was varied from 0.5 to 4.0 mg mL^{-1}. Error bars are ± one standard deviation (n=3).

Table 7-1: Batch binding and elution for pure β-Lac, pure LZY and a binary β-Lac and LZY mixture by in mixed mode MMM 1. Data shown is based on the average values ± one standard deviation (n=3).

Protein type	Lysozyme		β-Lactoglobulin	
Feed solution type	Single LZY	Binary LZY-β-Lac	Pure β-Lac	Binary LZY-β-Lac
Mass of membrane, mg	12.52 ± 0.11	12.65 ± 0.12	12.74 ± 0.14	12.65 ± 0.12
Feed protein, mg	1.00	1.00	1.00	1.00
Bound protein, mg	0.99 ± 0.00	0.92 ± 0.02	0.47 ± 0.02	0.57 ± 0.02
Capacity, mg protein g^{-1} membrane	79.22 ± 0.30	73.01 ± 0.55	36.76 ± 1.06	45.35 ± 0.98
Eluted protein, mg	0.93 ± 0.01	0.89 ± 0.02	0.36 ± 0.01	0.47 ± 0.01
Elution recovery *, %	93.8 ± 0.3	96.5 ± 1.3	76.1 ± 2.1	82.5 ± 1.6

* Elution recovery = mass of protein eluted/mass of protein bound × 100%

For the flow through mode using an isocratic salt elution, the elution data are given in tables 7-2 and 7-3, respectively, for β-Lac and LZY. The performance of mixed mode MMM 1 was also compared with its corresponding pure AEX MMM in Chapter 4 and pure CEX MMM in Chapter 5 prepared previously. The binding performance of the mixed mode membrane in flow through experiments was similar for pure or binary mixture protein solutions. However, the mixed mode MMM suffered from low elution recovery, especially for LZY.

LZY elution recovery was extremely low in flow through mode compared to batch mode. This was however, not experienced by the pure CEX MMM in flow through experiment. At present, there does not seem to be a strong reason why this decrease in elution recovery occurred only in the mixed mode MMM during flow through experiments. The possibility of LZY denaturation was hypothesized to occur in the mixed mode MMM in the presence of the anion resin. This denaturation would increase the contact area between LZY and the adsorbent surface and thus reduce the amount of protein desorbed (Avramescu et al. 2003a). If this happened, the contact time between the mobile phase and bound protein should be optimized in flow through experiments to allow enough time for LZY desorption.

Table 7-2: Flow through binding and elution for β-Lac in different feed solutions and different membrane types. The total β-Lac loaded onto the column was 2 mg and bound protein was eluted isocratically using 1 M NaCl salt. Data shown is based on the averages values ± one standard deviation (n=3).

Membrane type	AEX MMM	MMM 1	MMM 1
Feed solution type	Pure β-Lac	Pure β-Lac	Binary β-Lac – LZY
Mass of membrane, mg	91.25 ± 6.36	63.40 ± 0.58	63.40 ± 0.58
Adsorbent loading in the membrane, %	50	15	15
Bound protein, mg	1.83 ± 0.11	0.71 ± 0.13	0.74 ± 0.06
Capacity, mg protein bound g^{-1} membrane	20.02 ± 0.35	11.20 ± 2.01	11.75 ± 1.07
Normalized capacity, mg protein bound g^{-1} adsorbent	40.16 ± 0.71	74.68 ± 13.38	78.30 ± 7.10
Eluted protein, mg	1.70 ± 0.08	0.52 ± 0.01	0.54 ± 0.02
Elution recovery, %	92.8 ± 1.3	75.7 ± 16.2	73.0 ± 4.5

Good accessibility of protein into the adsorbent in the mixed mode membrane matrix was indicated by its high normalized protein capacity value. However, the direct comparison with pure anionic or cationic membranes in table 7-2 and 7-3 were not feasible. The maximum capacity for pure AEX or CEX MMMs was still below its real capacity value due to limitation of protein loaded into the column. In this pure membrane, almost all 2 mg of the protein loaded was bound by the membrane.

In mixed matrix interaction mode, both acidic and basic proteins were demonstrated to bind simultaneously onto the membrane. In theory, it would have been possible to elute bound acidic protein by decreasing the pH of the system, while bound basic protein could be eluted by increasing the pH. Table 7-4 shows the recovery of acidic protein eluted by decreasing the pH to pH 4 and then using 1 M NaCl for eluting the remaining bound protein in the membrane. A typical chromatogram from this protein elution protocol is shown in figure 7-9.

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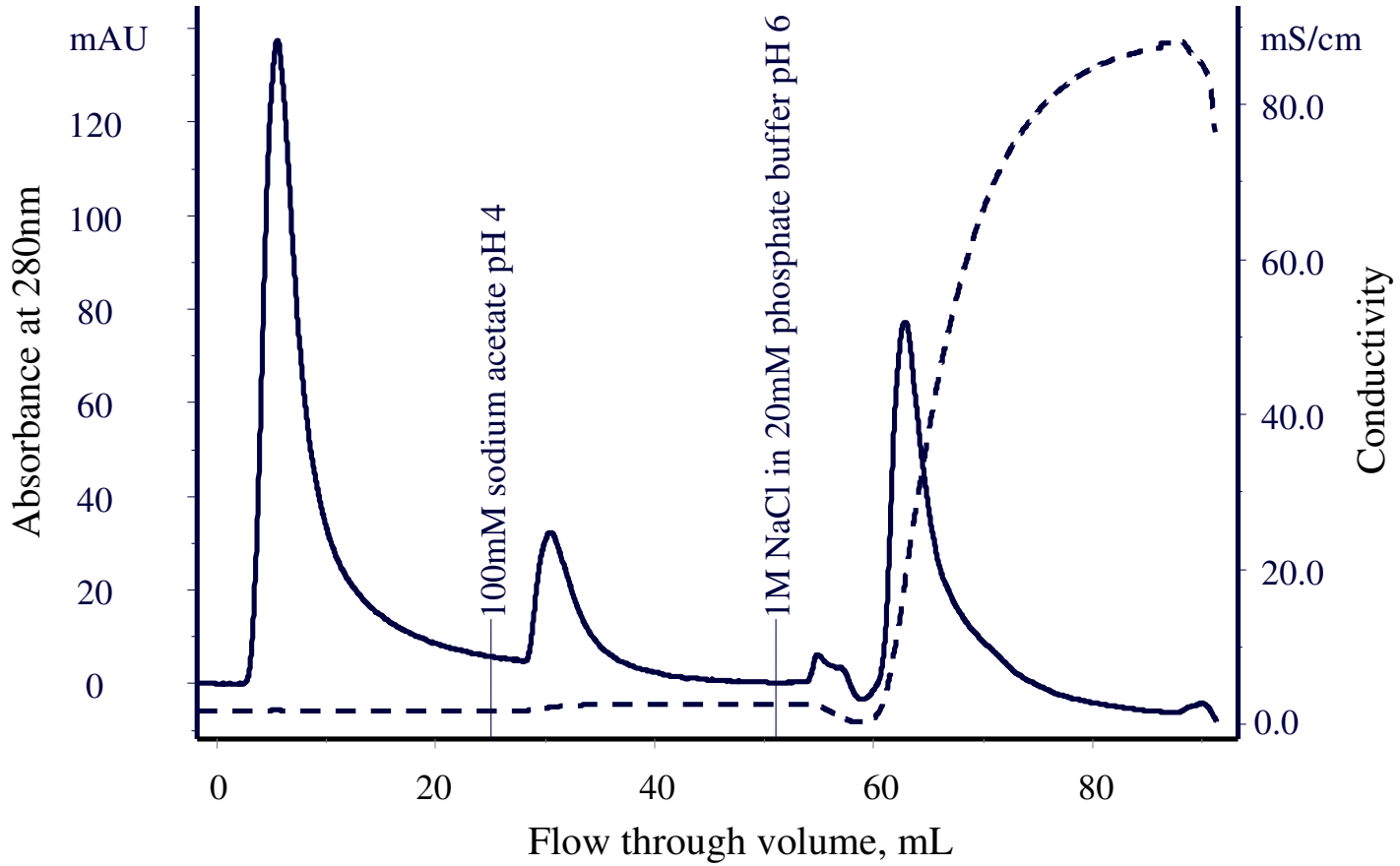


Figure 7-9: Typical chromatogram for elution of binary LZY and β-Lac using pH elution followed by 1 M NaCl elution.

Table 7-3: Flow through binding and elution for LZY in different feed solutions and membrane types. Total LZY loaded onto the column was 2 mg and bound protein was eluted isocratically using 1 M NaCl. Data shown is based on the averages values ± one standard deviation (n=3).

Membrane type	CEX MMM	MMM 1	MMM 1
Feed solution type	Pure LZY	Pure LZY	Binary LZY – β-Lac
Mass of membrane, mg	92.08 ± 11.70	63.40 ± 0.58	63.40 ± 0.58
Adsorbent loading in the membrane, %	30	15	15
Bound protein, mg	1.94 ± 0.05	1.45 ± 0.05	1.51 ± 0.02
Capacity, mg protein bound g^{-1} membrane	22.75 ± 2.33	22.80 ± 0.72	23.81 ± 0.57
Capacity, mg protein bound g^{-1} adsorbent	75.83 ± 7.70	152.00 ± 4.79	158.72 ± 3.80
Eluted protein, mg	1.70 ± 0.14	0.93 ± 0.09	0.89 ± 0.03
Elution recovery, %	87.6 ± 6.4	64.3 ± 7.7	59.2 ± 1.2

Total protein elution recovery was still low using this two-step protocol, similar to the experience with the previous protocol using salt elution. However, two protein fractions could be recovered in high purity by this combination of pH and salt elution. The first pH elution fraction contained almost 87% of the target acidic protein β-Lac, whereas about 85% of LZY was present in the salt elution fraction as shown in table 7-4.

7.3.5 Whey fractionation

7.3.5.1 Batch fractionation

Tables 7-5 (a) and 7-5 (b) show batch fractionation data for the mixed mode MMM 2 using whey and LF-spiked whey, respectively. In figure 7-10, the SDS-PAGE gel from each step of the fractionation process was visualized. Among the acidic whey

proteins, β-Lac has a binding preference onto this membrane. The second major acidic protein, α-Lac, also bound to a small extent but most was leached out during the washing step, as shown in lanes 4 and 8 in figure 7-10. According to the literature, the binding strength of the three major proteins on anion exchanger could be postulated to be in the order: β-Lac > BSA > α-Lac (Goodall et al. 2008; Weinbrenner and Etzel 1994). In fact, in some cases, β-Lac could displace other positively proteins bound onto the anionic membrane (Goodall et al. 2008). All LF in 1 mL of whey was bound onto MMM 2. However, a low elution recovery was obtained using the mixed mode MMM for whey fractionation.

Table 7-4: Elution recovery of LZY and β-Lac at different pH of elution in flow through experiment for mixed mode MMM 1. Data shown is based on the averages values ± one standard deviation (n=3).

Protein type	LZY	β-Lac
Feed protein, mg	2.00	2.00
Bound protein, mg	1.53 ± 0.04	0.72 ± 0.06
	Elution with 100 mM sodium acetate pH 4	
Eluted protein, mg	0.07 ± 0.01	0.39 ± 0.01
Elution recovery, %	4.7 ± 0.9	54.6 ± 2.9
Fraction purity, %	15.4 ± 2.3	86.6 ± 1.0
	Elution with 20 mM sodium phosphate pH 6, 1 M NaCl	
Eluted protein, mg	0.69 ± 0.03	0.11 ± 0.01
Elution recovery, %	45.3 ± 2.4	14.7 ± 0.7
Fraction purity, %	84.6 ± 2.3	13.4 ± 1.0
Total recovery, %	50.0 ± 3.1	69.3 ± 2.7

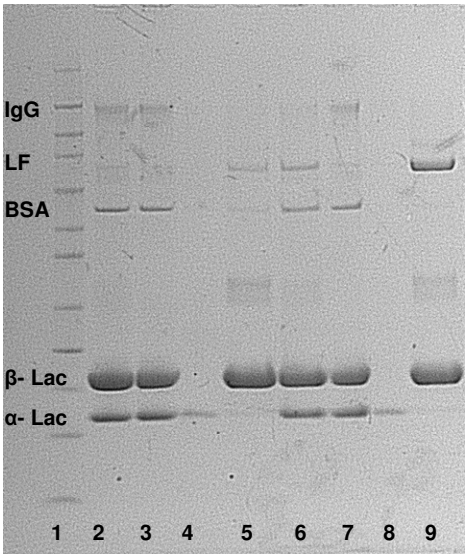


Figure 7-10: SDS-PAGE gel for batch whey fractionation by mixed mode MMM 2. Lane 1 – marker, normal whey fractionation: lane 2 – whey (4X dilution), lane 3 – unbound fraction (4X dilution), lane 4 – washing fraction, lane 5 – elution fraction, LF-spiked whey: lane 6 – LF-spiked whey (4X dilution), lane 7 – unbound fraction (4X dilution), lane 8 – washing fraction, lane 9 – elution fraction.

7.3.5.2 Cross-flow fractionation

A plate-and-frame module was used to increase the area of the membrane for whey fractionation. Compared to the filter holder module in flow through mode (15 cm^2), the plate-and-frame module area was 50 cm^2 and able to accommodate several layers of membrane in the module. However, when using a plate-and-frame module operated in cross-flow approach, protein losses and/or dilution can occur due to the large tubing volume of the system (permeate side, retentate side, recycle tubing, etc). Figure 7-11 shows a typical chromatogram run on the cross-flow filtration system for whey fractionation. The protein recovered in the final elution fraction is given in table 7-6 and confirmed by an SDS-PAGE gel in figure 7-12.

β-Lac, LF and IgG were recovered by the membrane from the cross-flow experiment. Because the run was operated under non-optimized conditions, a low yield resulted. In order to get a better yield, the amount of whey loaded onto the system, the number of the membranes in the module and the cross-flow operating parameters need to be optimized. However, such optimization was beyond the scope of the present work.

Table 7-5: Batch binding of mixed mode MMM 2 for (a) whey and (b) LF-spiked whey. 1 mL of whey was incubated with 2.64 cm^2 of membrane at pH 6 and elute with 1 M NaCl in 20 mM sodium phosphate pH 6. Mass of membrane used was 18.53 ± 0.64 mg. Data shown is based on the averages values ± one standard deviation (n=3).

(a) Whey

	α-Lac	LF	BSA	β-Lac	IgG
Protein feed to the system, mg	1.344	0.027	0.085	3.631	0.509
Protein bound to the membrane, mg	0.058 ± 0.010	0.027 ± 0.000	0.017 ± 0.017	0.988 ± 0.015	0.057 ± 0.009
Capacity, mg protein bound g^{-1} membrane	3.15 ± 0.49	1.47 ± 0.03	0.92 ± 0.94	54.08 ± 0.71	3.08 ± 0.52
Capacity, mg protein bound cm^{-2} membrane	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.01	0.37 ± 0.01	0.02 ± 0.00
Elute protein, mg	0.008 ± 0.001	0.017 ± 0.003	0.000 ± 0.000	0.756 ± 0.089	0.040 ± 0.004
Recovery, % (protein elute/protein bound)	14.89 ± 2.66	61.88 ± 10.18	0.00 ± 0.00	76.47 ± 7.90	72.58 ± 18.49

(b) LF-spiked whey

	α-Lac	LF	BSA	β-Lac	IgG
Protein feed to the system, mg	1.383	0.241	0.070	3.762	0.570
Protein bound to the membrane, mg	0.131 ± 0.042	0.214 ± 0.007	0.000 ± 0.000	1.108 ± 0.150	0.128 ± 0.027
Capacity, mg protein bound g^{-1} membrane	7.16 ± 2.24	11.44 ± 0.73	0.00 ± 0.00	59.21 ± 9.90	6.79 ± 1.11
Capacity, mg protein bound cm^{-2} membrane	0.05 ± 0.02	0.08 ± 0.00	0.00 ± 0.00	0.42 ± 0.06	0.05 ± 0.01
Elute protein, mg	0.004 ± 0.005	0.134 ± 0.006	0.000 ± 0.000	0.744 ± 0.043	0.052 ± 0.001
Recovery, % (protein elute/protein bound)	3.45 ± 4.87	62.56 ± 2.77	0.00 ± 0.00	68.26 ± 12.46	41.97 ± 8.44

Table 7-6: Binding properties of mixed mode MMM 2 for 30 mL of whey (LF spiked) in a cross flow system. Data shown is based on the averages values ± one standard deviation (n=3).

Protein	Protein feed to the system, mg	Protein in elution fraction, mg	Yield, % (protein elute/protein feed)
α-Lactalbumin	42.88	0.00 ± 0.00	0.00 ± 0.00
Lactoferrin	8.71	1.26 ± 0.19	14.46 ± 0.10
Bovine serum albumin	2.65	0.00 ± 0.00	0.00 ± 0.00
β-Lactoglobulin	119.86	20.80 ± 2.67	17.35 ± 2.23
Immunoglobulin	22.14	1.81 ± 0.17	8.16 ± 0.75

7.3.5.3 Flow through fractionation

In order to get better productivity estimation for the mixed mode interaction membrane, a flow though fractionation experiment at different volumes of whey was conducted. The aim was to find the volume at which all interesting proteins in whey were bound and eluted by the mixed mode MMM 2. According to figure 7-13, using three layers of membrane in a small filter holder (total membrane volume 0.91 mL), 0.75 mL of whey was completed bound and all proteins could be detected in the elution fraction.

For productivity estimation, 1,000 m^2 of mixed mode MMM would give a membrane volume of 200 L based on the 200 μm membrane thickness. Based on the above capacity, this membrane volume could bind total proteins from about 165 L of whey, which corresponds to 1.08 kg of total whey protein (6.55 g total protein per 1 mL whey). If we allow for a typical chromatographic cycle of 5 CV equilibration buffer, 165 L whey loading, 2 CV wash, 1 CV elution and 2 CV regeneration, this amounts to 2,165 L of permeate per cycle. At a modest 50 L m^{-2} h^{-1} permeate flux rate, this would have a total cycle time of approximately 2.6 minutes. Productivity based on membrane volume is therefore, 125 g $L^{-1}_{membrane}$ h^{-1}. A 1000 m^2 spiral-wound membrane module would typically have a hold-up volume of 1 m^3 (Cheryan 1998) so this productivity corresponds to 25 g L^{-1}_{module} h^{-1} on a membrane module volume

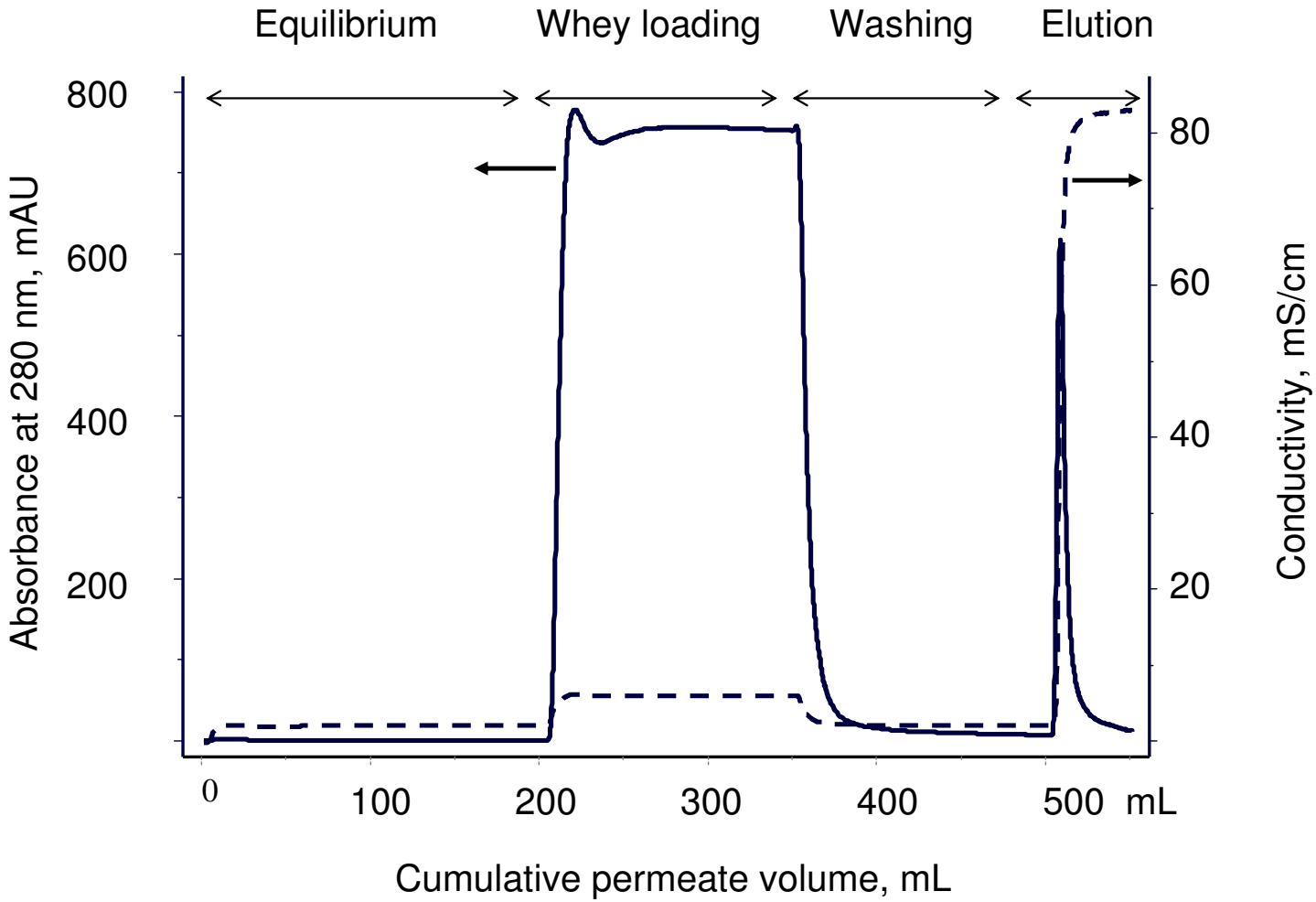


Figure 7-11: Typical chromatogram for cross-flow whey fractionation experiments in the AKTAcrossflow system.

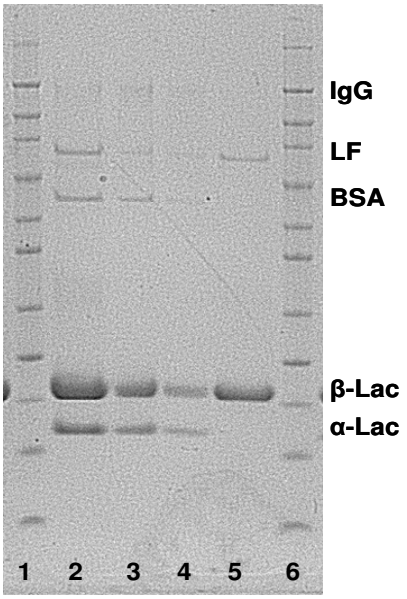


Figure 7-12: SDS-PAGE of several fractions from cross-flow fractionation of whey. Lane 1 – marker, lane 2 – LF-spiked whey (4X dilution), lane 3- unbound whey in retentate side (4X dilution), lane 4 – washing fraction in permeate side, lane 5 – elution fraction in elution side, lane 6 – marker.

basis or 74 g m^2 h^{-1} on a membrane area basis. These values compare well with reported values in the range 18 to 60 g L^{-1}_{resin} h^{-1} for laboratory-scale batch and packed-bed chromatography systems for whey protein isolate production (Doultani et al. 2003).

In figure 7-13, the bound protein on the membrane was eluted isocratically using 1 M NaCl in binding buffer. Different elution protocols were also tested, as shown in figures 7-14, 7-15 and 7-16 for qualitative visualization. In all experiments, 1 mL of LF-spiked whey was bound to the three layers of mixed mode MMM 2 in flow through mode. Based on these figures, single protein resolution was not achieved by the elution protocols tested. Further optimization of elution protocols is needed in future study to obtain good whey protein resolution during the elution step.

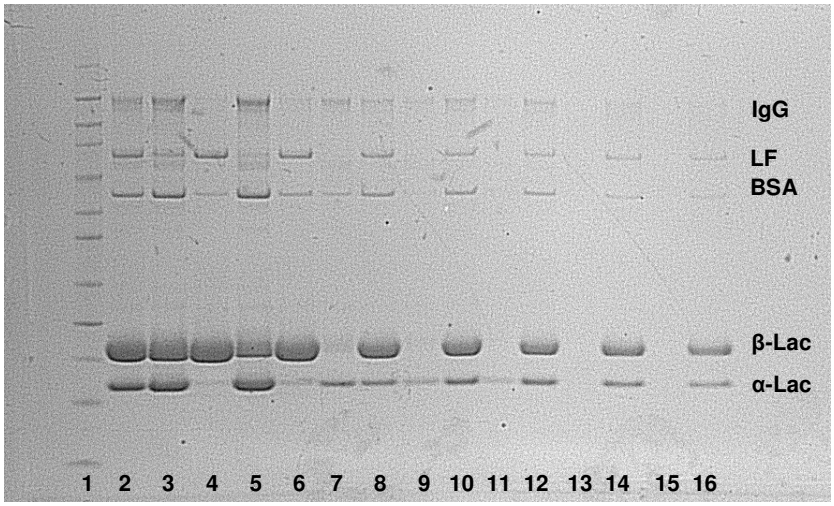
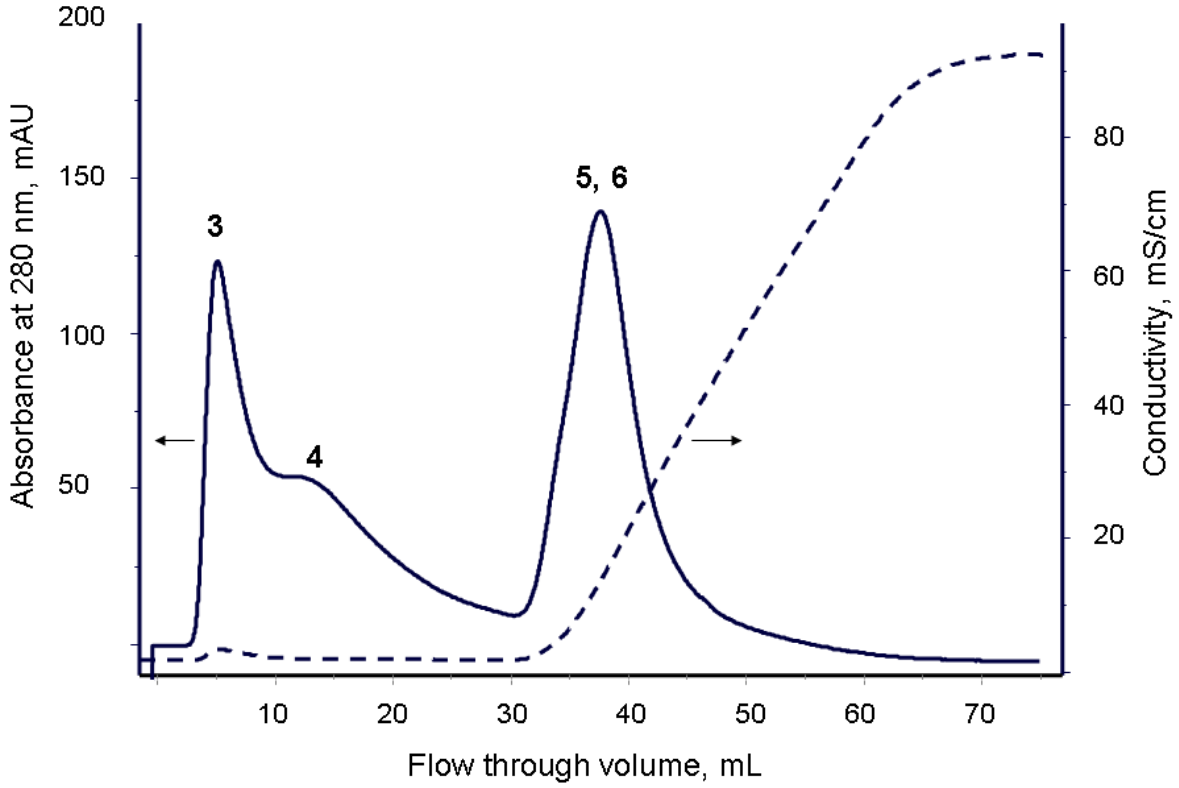


Figure 7-13: Unbound and elution fraction of whey proteins loaded at different injection volumes of whey into 3 layers of mixed mode MMM (mass 303.56 mg , diameter 44 mm, thickness 600 µm). Bound protein was eluted isocratically with 1 M NaCl in 20 mM sodium phosphate buffer pH 6. SDS-PAGE was run under non-reducing conditions. Lane 1 – marker, lane 2 – whey (4X dilution), lane 3 – unbound fraction (5 mL whey), lane 4 – elution fraction (5 mL whey), lane 5 – unbound fraction (3 mL whey), lane 6 – elution fraction (3 mL whey), lane 7 – unbound fraction (1.5 mL whey), lane 8 – elution fraction (1.5 mL whey), lane 9 – unbound fraction (1 mL whey), lane 10 – elution fraction (1 mL whey), lane 11 – unbound fraction (0.75 mL whey), lane 12 – elution fraction (0.75 mL whey), lane 13 – unbound fraction (0.5 mL whey), lane 14 – elution fraction (0.5 mL whey), lane 15 – unbound fraction (0.3 mL whey), lane 16 – elution fraction (0.3 mL whey).

7.4 Conclusions

Mixed mode interaction membrane chromatography was successfully developed using the concept of mixed matrix membrane preparation. The ratio of the different types of adsorptive resin incorporated into the membrane matrix can be tailor-made for protein recovery from a specific feed stream. The mixed mode cation and anion membrane chromatography developed was able to bind basic and acidic proteins simultaneously from a solution. The binding of target proteins by mixed mode MMM was not affected significantly by the presence both ion exchange charges in the membrane. However, the elution recovery of bound protein was relatively low, especially in flow through operation. A custom mixed mode MMM, consisting of

42.5 wt% of MP500 anion resin and 7.5 wt% SP Sepharose cation resin, was developed for binding all proteins from whey in a single pass. Based on the productivity estimation, this mixed mode MMM showed a productivity of 125 g total whey protein per L membrane per h.



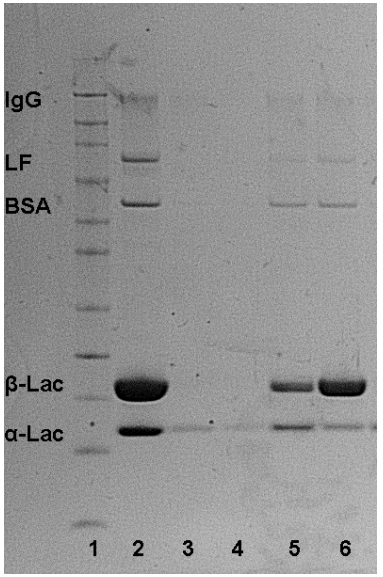
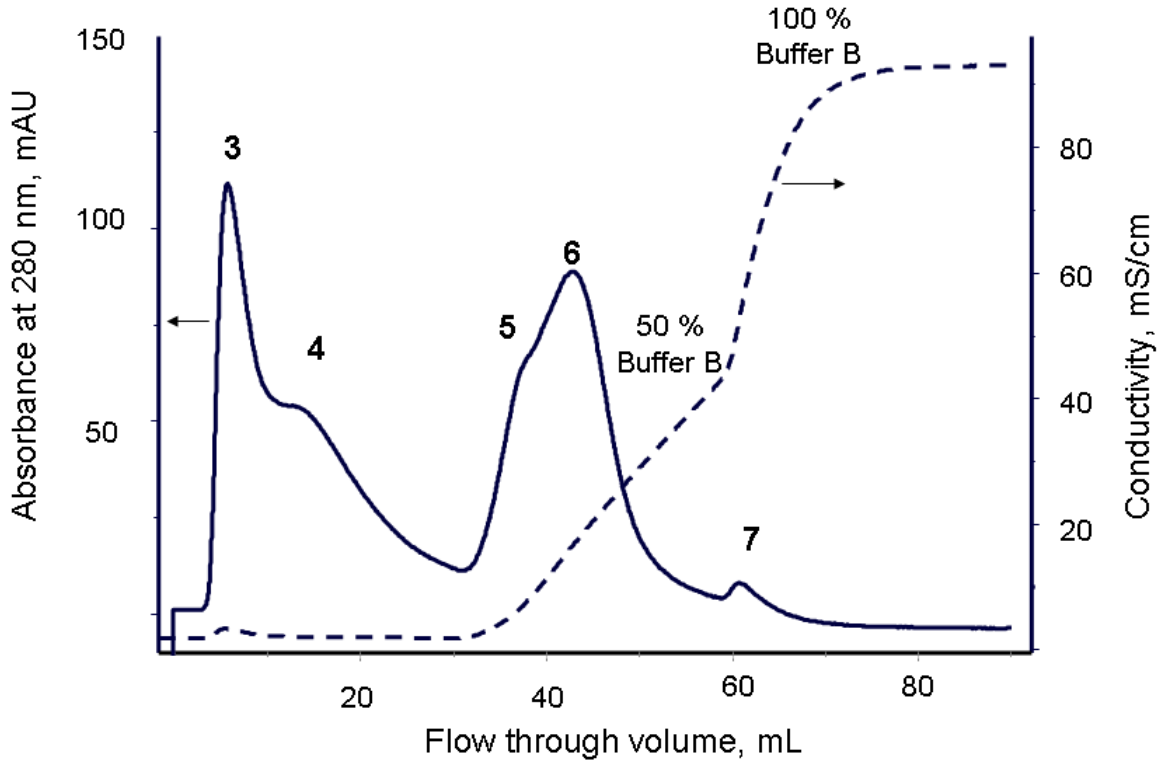


Figure 7-14: Linear elution gradient to 100 % buffer B. Lane 1 and lane 2 in the SDS-PAGE gel represent a marker and feed whey respectively.



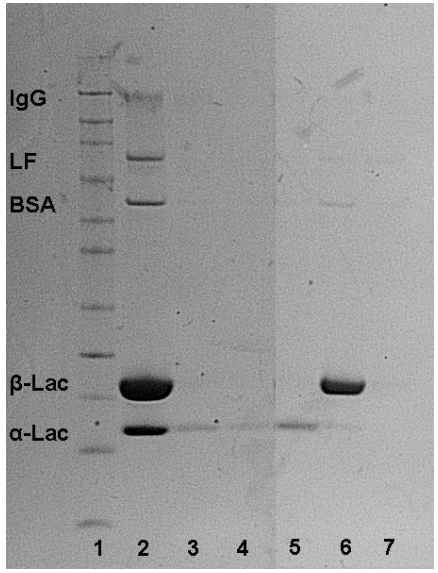


Figure 7-15: Linear elution gradient to 50% buffer B, followed by step elution at 100% buffer B. Lane 1 and lane 2 in the SDS-PAGE gel represent a marker and feed whey respectively.

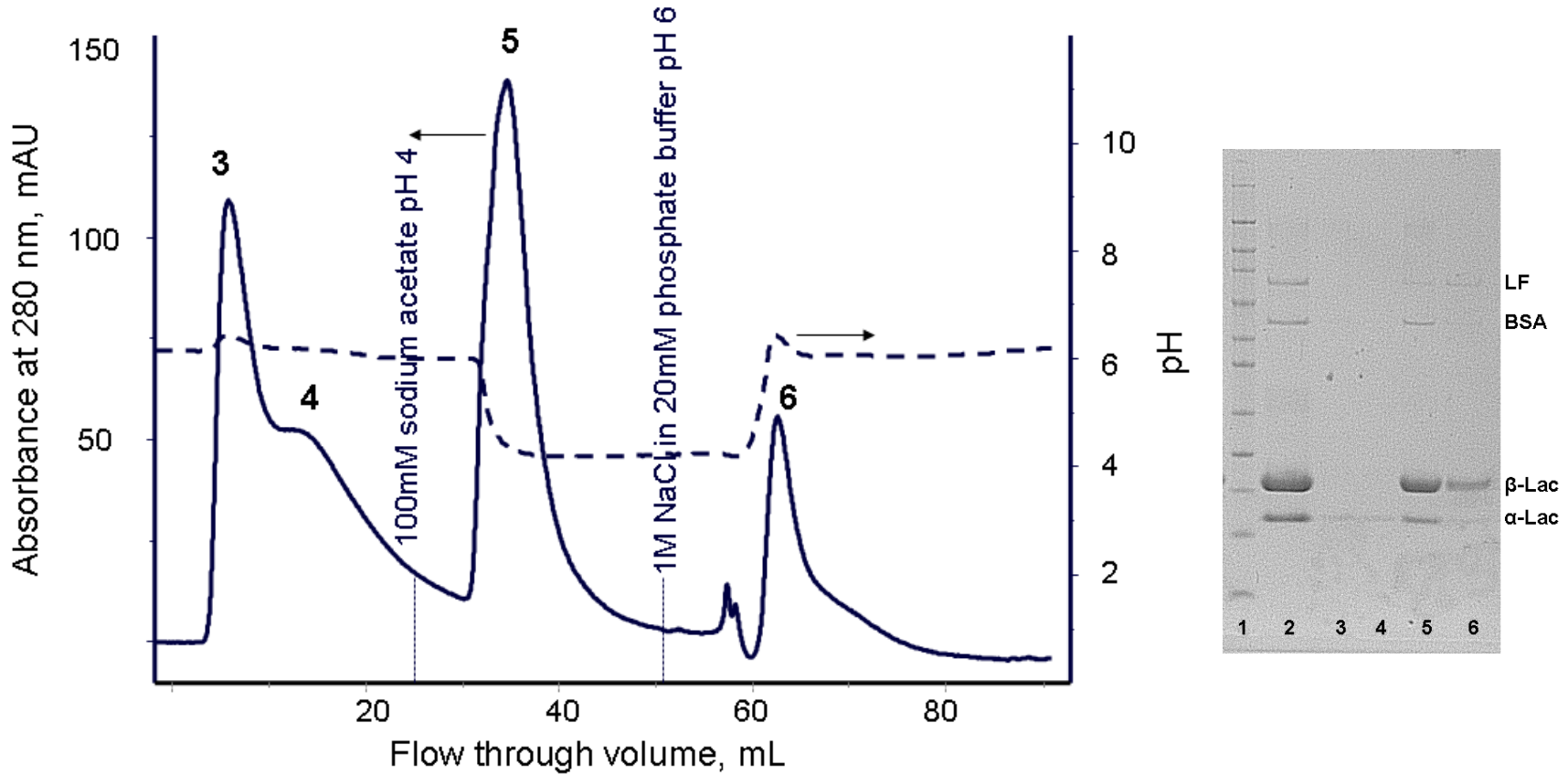


Figure 7-16: pH elution at pH 4 followed by step elution at 100% buffer B. Lane 1 and lane 2 in the SDS-PAGE gel represent a marker and feed whey respectively.

8 Conclusions and recommendations

8.1 Conclusions

Whey protein fractionation using membrane chromatography shows great potential for the dairy industry because the system depends mostly on the capacity of the adsorbent in the membrane without the need for complex column packing or limitations from column backpressures. The concept of MMM chromatography, which incorporates adsorptive particles during membrane casting, can be simply applied to prepare membrane chromatography and attain performances that are competitive with other membrane chromatography materials.

In the current study, anion exchange MMM chromatography was developed from EVAL base membrane using Lewatit MP500 as an anion exchange resin in the membrane matrix described in Chapter 4. The membrane was characterized in terms of structure and its static and dynamic binding capacities were measured. The optimum binding for β-Lac was found to be at pH 6.0 using 20 mM sodium phosphate buffer. The MMM had a static binding capacity of 120 mg β-Lac g^{-1} membrane (36 mg β-Lac mL^{-1} membrane) and 90 mg α-Lac g^{-1} membrane (27 mg α-Lac mL^{-1} membrane). In batch fractionation of whey, anion exchange MMM showed selective binding towards β-Lac compared to other proteins. The binding preference for whey protein component onto anion exchange MMM was demonstrated to follow this order: β-Lac > BSA > α-Lac. The dynamic binding capacity of β-Lac in whey solution was about 80 mg β-Lac g^{-1} membrane (24 mg β-Lac mL^{-1} membrane), which is promising and comparable for whey fractionation using this technology.

A cationic MMM was developed for recovery of LF from bovine whey by embedding ground SP Sepharose cation exchange resin into an EVAL polymer base membrane in Chapter 5. The static LF binding capacity of the cationic MMM was 384 mg g^{-1}membrane or 155 mg mL^{-1} membrane, exceeding the capacity of several commercial adsorptive membranes. Adsorption of LZY onto the embedded ion exchange resin was visualized by confocal laser scanning microscopy. The membrane chromatography system was operated in cross-flow mode to minimize fouling and enhance LF binding, resulting in an LF recovery as high as of 91%, with

high purity as seen by the band of sodium dodecyl sulfate polyacrylamide gel electrophoresis gel and the chromatogram of reversed phase chromatography. The system was operated at a constant permeate flux rate of 100 LMH, except during the whey loading step, which was run at 50 LMH. This is the first time a cross-flow MMM process has been reported for LF recovery from whey.

Hydrophobic interaction membrane chromatography was developed by incorporating Phenyl Sepharose resin into the membrane matrix. The Phenyl Sepharose MMM showed optimum whey protein binding at 2 M ammonium sulphate buffer at pH 6. However, at this salt concentration some nonspecific interaction of β-Lac and α-Lac occurred onto the EVAL base membrane. Maximum static binding capacity of Phenyl Sepharose MMM calculated by the Langmuir isotherm were 20.539 mg mL^{-1} of β-Lac, 45.573 mg mL^{-1} of α-Lac, 38.651 mg mL^{-1} of BSA and 42.046 mg mL^{-1} of LF. These values were comparable with the values reported in the literature. In flow through whey fractionation, the performance of phenyl sepharose MMM was similar to the HiTrap Phenyl column. However, Phenyl Sepharose MMM suffered from low elution recovery; most of whey protein component had an elution recovery less than 80% in step elution using a salt-free buffer. Linear gradient elution was also performed to resolve single whey protein components but no baseline separation was observed. This was the first attempt at producing a hydrophobic membrane using the mixed matrix concept in the membrane chromatography field.

For the first time, mixed mode interaction membrane chromatography was developed in a single membrane material using the mixed matrix preparation concept. 15 wt% of MP500 anion resin and 15 wt% SP Sepharose cation resin were added together to make a mixed mode interaction membrane. This membrane had a maximum static binding capacity of 41.877 g β-Lac g^{-1} membrane and 117.614 g LZY g^{-1} membrane. The membrane was successfully applied to bind acidic and basic proteins simultaneously from a mixture. A customized mixed mode MMM was also developed to achieve whey protein isolation in single pass, consisting of 42.5 wt% of MP500 anion resin and 7.5 wt% SP Sepharose cation resin. Both cross-flow and flow through operation was conducted for whey protein fractionation. Based on flow through experimental results, a productivity of 125 g whey protein L^{-1} membrane h^{-1} was calculated using this customized mixed mode MMM. By using the mixed matrix

preparation concept, the ratio of the different types of adsorptive resin incorporated into the membrane matrix can be tailor-made for protein fractionation from specific feed streams, offering versatility in preparing novel and efficient membrane chromatography materials.

8.2 Recommendations for future work

In the present study, EVAL was used as a membrane base polymer at a fixed mass percentage of 15 wt% in DMSO and 1-octanol. All types of mixed matrix membranes prepared in this study were based on this composition. However, depending on the type of resin to be incorporated into the membrane matrix, there is a limitation of the amount of resin that can be loaded. As an example, due to the viscosity factor, only 20% of SP sepharose cation resin can be added into EVAL polymer solution compared to 50% of Lewatit MP500 anion resin. Therefore, the base polymer composition should be optimized for each type of resin used for making different types of MMM chromatography materials. This will lead to the optimum resin loading capacity and at the same time may produce better membrane structure and properties.

Established polymers for membrane materials, especially hydrophilic polymers like cellulose, chitosan, polyamide, etc., can also be a good candidate as a base polymer for preparing MMM chromatography. At the same time, searching for different types of resin from different manufacturers should be continued in order to find a suitable resin with lower cost but comparable performance for protein binding. In terms of membrane configuration, the possibility to produce MMM chromatography in hollow fiber form for whey protein fractionation should be tested, to get the benefit of high surface area to volume ratio offered by this configuration.

It was demonstrated in this study that the cross-flow mode offered a new alternative to operate membrane chromatography in a more promising way for whey protein fractionation. Unlike in column chromatography, which generally loads a single pulse of feed in a single pass, the flow through fraction (i.e. retentate and permeate) has a chance to re-load onto the column during the whey loading step to enhance the protein binding during cross-flow operation. However, the cross-flow condition and

process parameters need to be optimized to fully utilize the potential of membrane chromatography using this operation mode.

The mixed mode interaction MMM prepared in Chapter 7, has the ability to adsorb both acidic and basic protein simultaneously. A proper elution strategy is necessary in order to elute the target protein of interest in an effective way. pH gradient elution might be one of the potential ways to obtain the bound protein from this kind of mixed mode MMM. Another type of mixed mode interaction membrane such as combination of hydrophobic and ionic interaction mechanisms may also be of interest for further membrane development. Unique separation ability was predicted by this type of membrane chromatography, especially for the protein separation that was previously governed by hydrophobic interaction chromatography. The salt concentration required for binding might be less by using this kind of mixed mode membrane compared to the same separation process using a hydrophobic interaction chromatography.

The use of CLSM to visualize the protein bound within the membrane structure should be extended to mixed mode interaction MMM developed in this study in Chapter 7. By labelling basic and acidic proteins with different fluorescent dyes, we would be able to see the binding pattern in mixed mode interaction membranes using CLSM. This would help to explain the behaviour of protein binding and assist in developing an optimizing structure for mixed matrix membrane chromatography. Furthermore, the distribution of anion and/or cation resin inside the membrane matrix could be monitored using different types of ionic dyes.

Mixed matrix membrane chromatography developed in the present study focused mainly on whey protein fractionation. Further exploration for different applications or feed stream is necessary in future work. In the current study, all whey fractionation processes were done at a lab scale, therefore pilot scale and scale up studies need to be done in further research.

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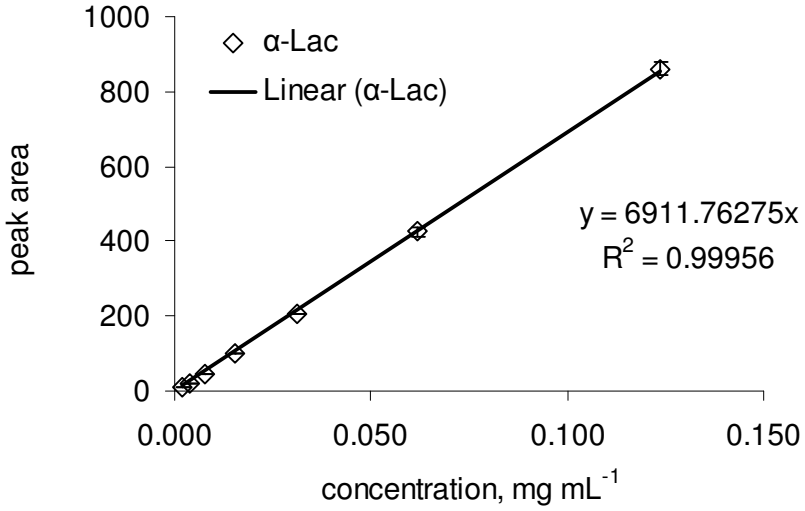
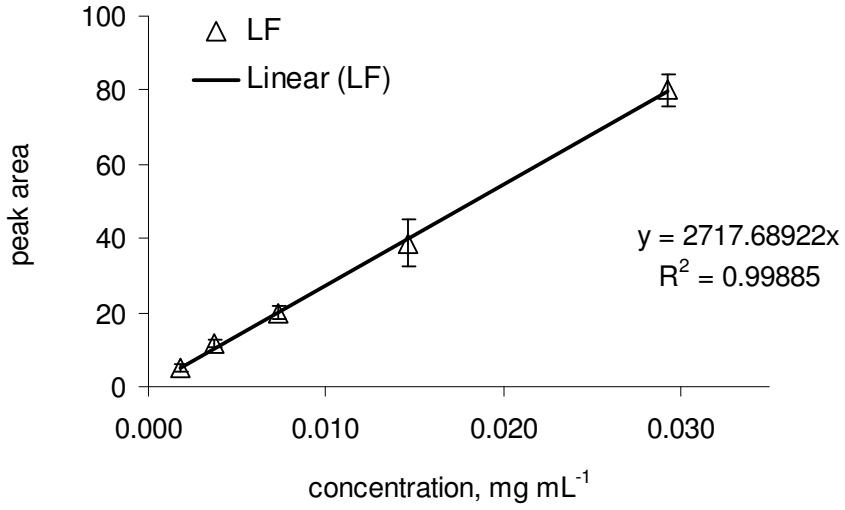
Appendices

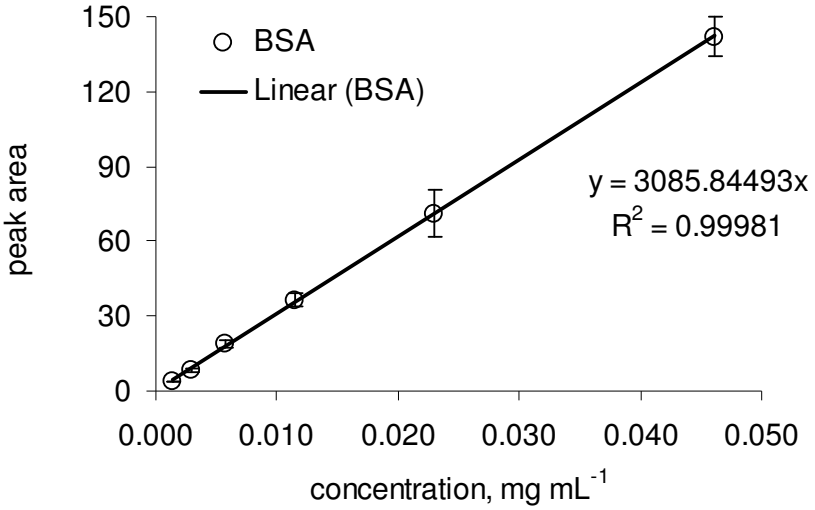
Appendix 1: Whey protein assay using 1 mL Resource RPC column according to protocol in section 3.8.



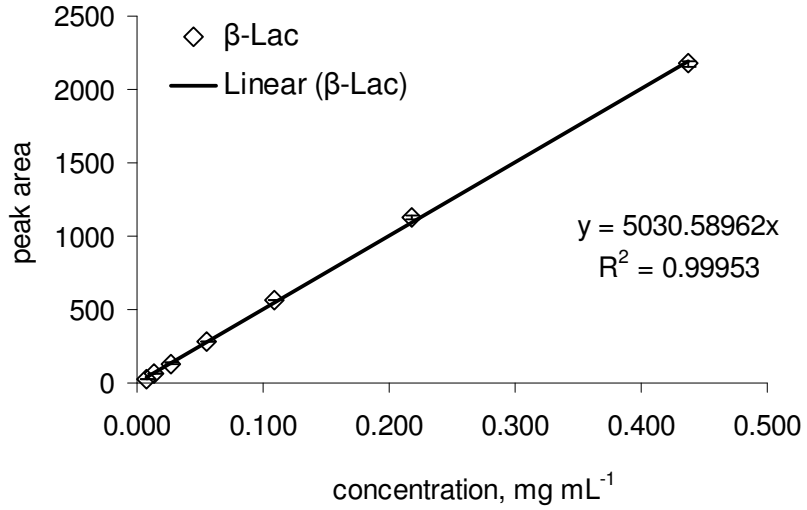
(a) standard curve for α-lactalbumin



(b) standard curve for lactoferrin

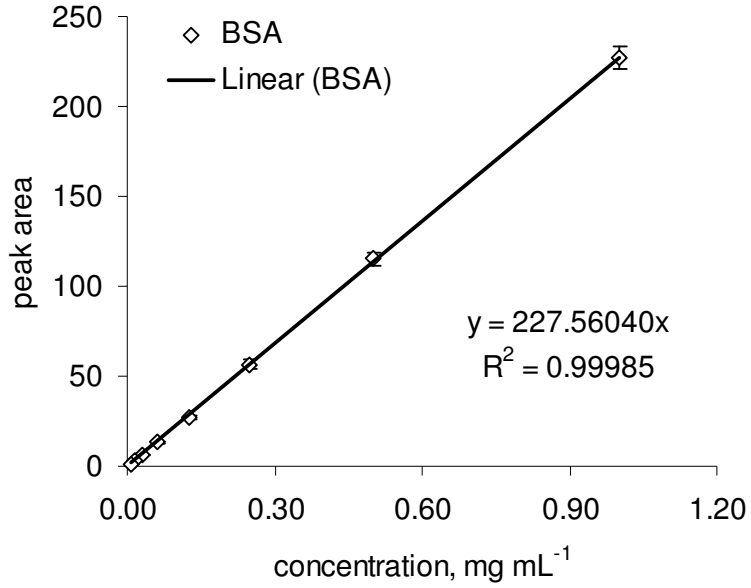


(c) standard curve for bovine serum albumin

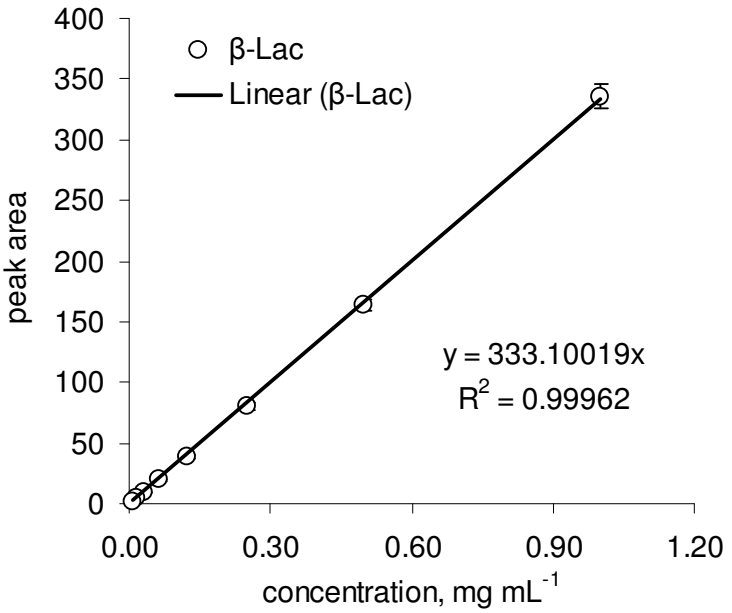


(d) standard curve for β-lactoglobulin

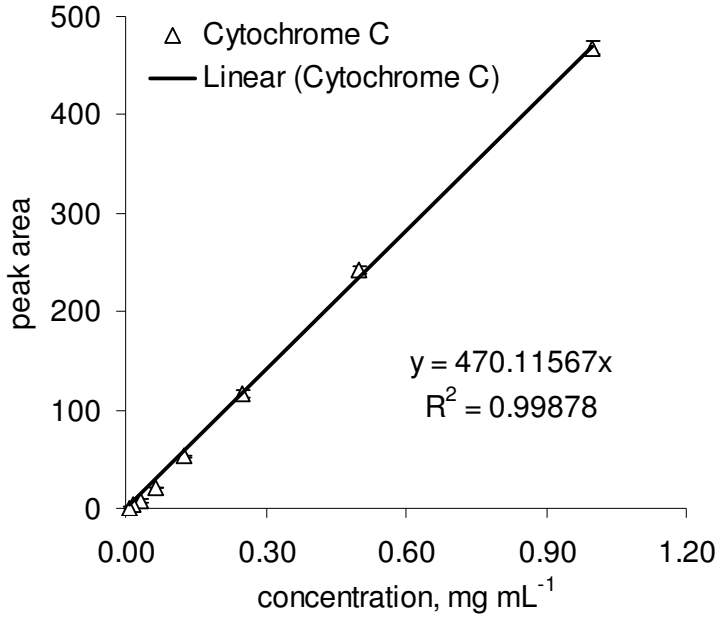
Appendix 2: Quaternary protein assay using 1 mL Resource RPC column according to protocol in section 7.2.6.1.



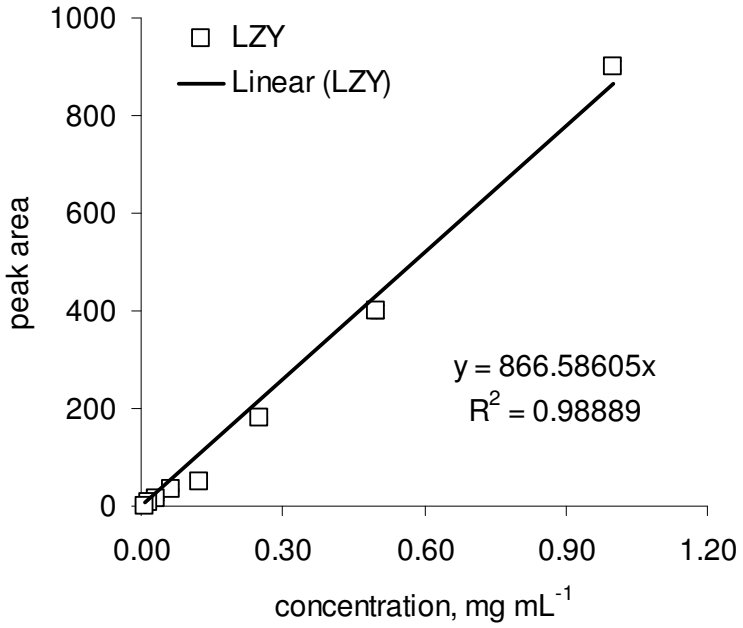
(a) standard curve for bovine serum albumin



(b) standard curve for β-lactoglobulin

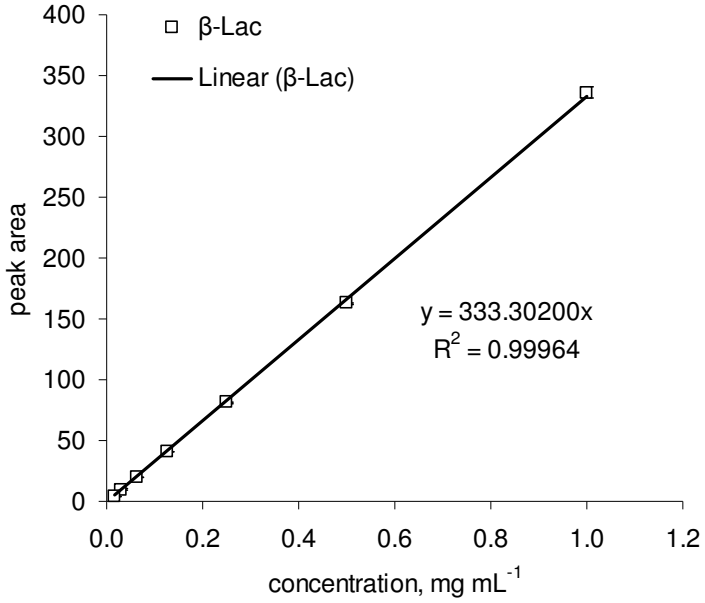


(c) standard curve for cytochrome C

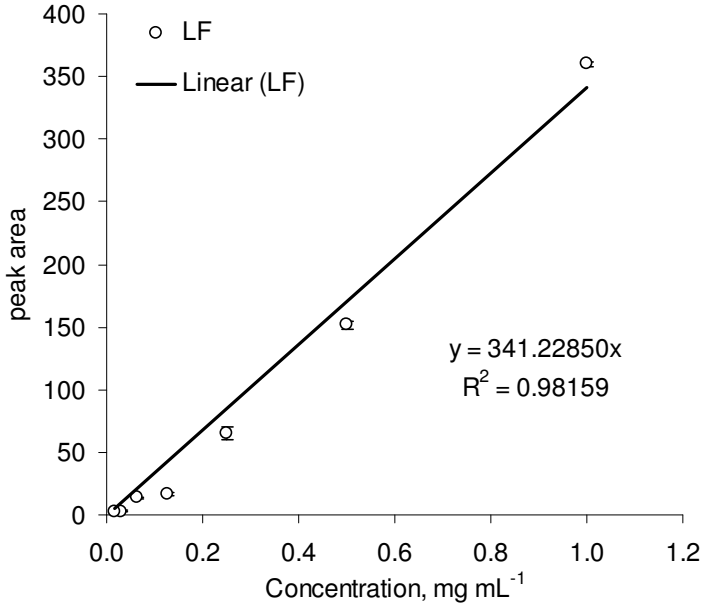


(d) standard curve for lysozyme

Appendix 3: Binary β-Lac and LF protein assay using 1 mL Resource RPC column according to protocol 7.2.6.2.



(a) standard curve for β-lactoglobulin



(b) standard curve for lactoferrin